EDITORIAL

Polio eradication: next steps and future challenges
Maria Zambon and Javier Martin

RAPID COMMUNICATION

Multidrug-resistant Neisseria gonorrhoeae isolate, belonging to the internationally spreading Japanese FC428 clone, with ceftriaxone resistance and intermediate resistance to azithromycin, Ireland, August 2018
Daniel Golparian, Lisa Rose, Almida Lynam, Aia Mohamed, Beatrice Bercot, Makoto Ohnishi, Brendan Crowley and Magnus Unemo

SURVEILLANCE AND OUTBREAK REPORTS

Acute flaccid paralysis (AFP) surveillance: challenges and opportunities from 18 years' experience, Spain, 1998 to 2015
Josefa Masa-Calles, Nuria Torner, Noemí López-Perea, María de Viarce Torres de Mier, Beatriz Fernández-Martínez, María Cabrerizo, Virtudes Gallardo-García, Carmen Malo, Mario Margolles, Margarita Portell, Natividad Abadía, Aniceto Blasco, Sara García-Hernández, Henar Marcos, Núria Rabella, Celia Marín, Amelia Fuentes, Isabel Losada, Juan García Gutiérrez, Alba Nieto, Visitación García Ortúzar, Manuel García Cenoz, José María Arteagoitia, Ángela Blanco Martínez, Ana Rivas, Daniel Castrillejo and Spanish AFP Surveillance Working Group

Arbovirus surveillance: first dengue virus detection in local Aedes albopictus mosquitoes in Europe, Catalonia, Spain, 2015

RESEARCH ARTICLES

A comparison of two biological markers of recent hepatitis C virus (HCV) infection: implications for the monitoring of interventions and strategies to reduce HCV transmission among people who inject drugs
Vivian D Hope, Ross J Harris, Peter Vickerman, Lucy Platt, Justin Shute, Katelyn J Cullen, Samreen Ijaz, Sema Mandal, Fortune Ncube, Monica Desai and John V Parry

Impact of pneumococcal conjugate vaccines introduction on antibiotic resistance of Streptococcus pneumoniae meningitis in children aged 5 years or younger, Israel, 2004 to 2016
Shalom Ben-Shimol, Noga Givon-Lavi, David Greenberg, Michal Stein, Orli Megged, Avihu Bar-Yochai, Shahar Negari, Ron Dagan and on behalf of the Israel Bacteremia and Meningitis Active Surveillance Group
In 1988, the World Health Assembly resolved to eradicate polio. At that time, polio was endemic in 125 countries and paralyzed around 1,000 children per day. Since then, polio cases due to infection with wild poliovirus (WPV) have decreased by more than 99.9% from over 350,000 cases a year to 37 cases in 2016 and 22 in 2017 [1]. Of the three WPV serotypes, 1, 2 and 3, WPV2 has not been detected since 1999 and was declared eradicated in September 2015. This allowed a global switch from live trivalent oral polio vaccine (tOPV) to live bivalent oral polio vaccine (bOPV), eliminating the need for live type 2 poliovirus vaccine strains. At the same time, this switch has created a need for universal use of inactivated polio vaccine (IPV) to ensure immune protection against type 2 poliovirus.

Clinical and virological surveillance using the acute flaccid paralysis (AFP) case definition, in tandem with comprehensive vaccination programmes using OPV have been extremely successful in high disease burden countries. WPV3 was last detected in November 2012 in Nigeria, and since this time WPV1 has been the sole circulating WPV type globally. WPV transmission has persisted in only two countries: Afghanistan and Pakistan, although in August 2016 it was also detected in Nigeria [1]. As we begin to see the light at the end of the tunnel, a relentless focus on achieving complete vaccination coverage in the areas still using OPV, together with a global commitment to universal IPV coverage and diverse approaches to surveillance, are needed to achieve the final target.

Clinical surveillance
The assessment of polio elimination status in a country is based upon demonstration of routinely high uptake of vaccine in children and evidence of strong polio surveillance. One of the hallmarks of the smallpox elimination campaign in its final stages in the 1970s was relentless tracking and detailed investigations of possible cases, however difficult the circumstance or how improbable the clinical case. Smallpox had a distinct clinical presentation making it easier to recognize compared with polio, where the key indicator clinical syndrome for the elimination is acute flaccid paralysis (AFP). This occurs in less than 5% of poliovirus-infected individuals, and is also the result of poorly understood aetiologies such as Guillain-Barré syndrome. Currently,
many countries struggle to undertake AFP surveillance. Polio-associated AFP is a rare disease overall, and its declining incidence and lack of perceived importance has led to difficulties in use and verification of individual cases [3]. As discussed in this week’s Eurosurveillance report of the Spanish experience of AFP surveillance over the past 20 years, in at least a third to two thirds of cases the supporting virological investigations may also be less than optimum [9]. The findings emphasise that the overall sensitivity of passive AFP case finding, as a tool for detection of polio circulation in the era of eradication, is insufficient and needs to be supplemented. Awareness raising within the clinical and paediatric communities, of the importance of timely notification of possible AFP cases and detailed and disciplined investigation, is necessary to overcome the presumption that polio has indeed disappeared and to dispel the notion that case follow-up effort is no longer required. Inadequate sampling for virological investigation is a risk to clinical surveillance programmes. The widest range of samples, including respiratory and faecal samples, should be analysed from each possible AFP case as part of a carefully coordinated approach to thoroughly investigate causality. Virological analysis of body fluids from AFP cases and other polio-compatible neurological illnesses is a crucial tool in verifying the lack of circulating polioviruses. The gradual shift in clinical practice towards investigating possible infectious episodes of acute onset neurological illness using PCR with cerebrospinal fluid (CSF) as the specimen of choice will further reduce the sensitivity of AFP surveillance. Using well-timed faecal samples for virological investigations, as recommended in the WHO Polio laboratory manual [10], provides a much higher chance to detect poliovirus infection.

Correct classification of poliovirus isolates as vaccine-like strains, VDPVs and WPVs, through a competent laboratory network, is essential to support rapid and thorough outbreak investigations and to help orchestrate the best public health response. Tried and tested classical laboratory methodologies involving selective cell culture systems for virus isolation followed by molecular typing methods are very sensitive for the detection of WPV and VDPV strains but, as yet, fully validated direct detection methods for poliovirus identification in stool extracts are not available. As mentioned above, WPV1 is still circulating in areas of Pakistan and Afghanistan. In addition, cVDPVs are still causing outbreaks in various countries of Africa (Democratic Republic of Congo, Nigeria, Niger and Somalia) and in Papua New Guinea [1]. Until poliovirus transmission is interrupted, all countries remain at risk of importation of PV. Especially vulnerable are countries with inadequate public health and immunisation services and travel or trade links to endemic countries. With the eradication of WPV2, it is particularly important to focus on rapid response to cVDPV2 outbreaks and to ensure there is heightened biosecurity for WPV2 held in laboratory facilities.

Environmental surveillance
Work has been undertaken to develop alternative surveillance approaches to verify that there are no circulating polioviruses. This includes virus isolation and detailed molecular characterisation of enteroviruses recovered from severe, neurological illness cases [11], and environmental surveillance (ES) of waste water and sewage systems [12]. Unlike clinical (AFP) surveillance, ES can monitor large populations using smaller numbers of samples and detect the introduction of poliovirus even before the appearance of AFP cases, and can be a sensitive tool to pick up circulation of WPV or VDPV. The silent transmission of type 1 WPV in 2013 in Israel has demonstrated the value of active ES that can detect poliovirus excretion in the population in the absence of polio-related AFP cases [13]. While the application of advanced molecular genomics tools for the analysis of complex environmental samples will add sophistication and improve current analytical approaches [14], there are substantial logistical and financial constraints in implementing ES at scale in densely populated countries. Such approaches require detailed consideration of sampling strategies and cross sectoral collaborations involving parties outside the primary health system. Implementation of ES programmes in a rational way will support strengthened clinical surveillance programmes, though much more work is needed to refine sampling strategies and develop standardised sampling and laboratory analytical methods.

Biocontainment
As the focus of the global eradication programme shifts away from tracking the transmission of WPV towards detailed oversight of poliovirus-related laboratory and manufacturing activities at national level, there is now a need to give greater emphasis to the regulation of biocontainment. The report in 2017 of two accidental exposures to WPV-2 in a Dutch manufacturing plant [15] was a wake-up call on what might be the consequences of breaches in biocontainment or gaps in scenario planning and emphasised the need for particular vigilance in these facilities worldwide. The incident also highlighted the necessity of having detailed public-health risk-management plans ready for uncontrolled and unintended release events arising in laboratory environments. This aspect has been addressed in Europe by detailed simulation exercises led by WHO in autumn 2018 to test national plans and preparedness for such events. In addition, recent reports of tOPV use in India that led to the exposure of children to live type 2 vaccine strain after the tOPV to bOPV switch [16,17] showed that stringent regulatory oversight of vaccine manufacturing facilities is absolutely required. The risks associated with poliovirus vaccine production, including IPV, are now magnified in the absence of circulating viruses. These include the inadvertent release of large volumes of biological materials, potentially containing high titres of live poliovirus to the sewage system, as part of the IPV vaccine production process. When this happened on a previous occasion,
it required a major public health response and complex investigations to assess the impact of possible large-scale environmental contamination [18].

**Polio essential facilities**

Under the WHO GAP III [6], it is envisaged that the number of facilities holding and working with WPV will decrease markedly. Countries will be required to establish a national authority for containment (NAC) by the end of 2018, to register as polio essential facilities (PEFs) organisations handling poliovirus within their borders and to provide oversight of their activities. NACs will scrutinise critical testing functions that require the use of live poliovirus much more stringently. These will include vaccine manufacture and laboratory activities, such as measuring population immunity and immunogenicity testing and preparation of standards and controls for diagnostic assays. The risks associated with poliovirus vaccine production, including IPV, are now magnified in the absence of circulating viruses, as manufacturing facilities will be the single largest source of live poliovirus. Implementation deadlines for registration of PEFs and enhanced regulation of biocontainment appear challenging, but the necessity of increased regulatory oversight at this stage of the end game, while recognising the need to ensure global IPV availability, is uncontroversial, even without the examples above.

During the coming 12 to 18 months, the work of National Accreditation Committees should generate a greater understanding of the distinction between the activities that require a PEF and how to classify and handle poliovirus infectious and potentially infectious materials (IMs and PIMs) [19]. The committees will support GAP III processes and serve to strengthen biological risk management in facilities delivering critical functions. Global research priorities for a virus that is being eradicated link directly to risk reduction associated with polio vaccine manufacture, where there is no alternative to the use of live virus. Some of the biocontainment risk associated with continuing use of OPV could be addressed through the use of safer, genetically stabilised, live-attenuated vaccine strains. Similarly, the use of hyperattenuated poliovirus strains such as (S19) for vaccine production, quality assurance programmes and immunogenicity testing would improve risk management during the IPV vaccine manufacturing process [20]. These and other related research activities will require the use of live poliovirus strains as gold standards for some years to come, with the expectation that ultimately the fruits of the labour will reduce the global biorisk of polio vaccine manufacture.

We have come a long way since the March of Dimes was launched almost 100 years ago in the United States as the first popular societal movement against polio. Today’s achievements in polio eradication arise from the combined and collaborative efforts of many different international health agencies, private foundations and governments and individuals being vaccinated, each playing their part. Taking a backwards look is a reminder of the scale of the challenge and the immense human effort that has been required to get us to where we are now. With the end in sight, the final push towards complete polio eradication, to overcome the remaining challenges, requires a last heave and concerted effort from many different sectors to finally eliminate the scourge of polio-associated paralysis.

**Conflict of interest**

None declared.

**Authors’ contributions**

The manuscript was conceived and jointly written by both authors, who have both participated in drafting and correcting the manuscript at all stages.

**References**


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We describe a multidrug-resistant *Neisseria gonorrhoeae* urethritis case with ceftriaxone resistance and azithromycin intermediate resistance in a heterosexual man in Ireland, August 2018. Whole-genome sequencing showed that the isolate IR72 belongs to the internationally spreading multidrug-resistant ceftriaxone-resistant FC428 clade, initially described in Japan in 2015. IR72 was assigned MSLT ST1903, NG-MAST ST17842 and NG-STAR type 1133, including the ceftriaxone resistance-mediating penA-60.001. Global awareness of spreading ceftriaxone-resistant gonococcal strains that threaten recommended dual therapies is essential.

We report the detailed characterisation of the first multidrug-resistant (MDR) *Neisseria gonorrhoeae* isolate with ceftriaxone resistance and intermediate resistance to azithromycin causing urethritis in a heterosexual male in Ireland in 2018. We show using whole genome sequencing (WGS) that the Irish isolate belongs to the internationally spreading MDR and ceftriaxone-resistant FC428 clone, initially described in Japan in 2015, which is further evolving [1-5].

**Case description**

In August 2018, a heterosexual male presented to specialised sexually transmitted infection (STI) services in Ireland with symptoms of urethral discharge and dysuria. He reported having recent sexual contact with a female during a visit to a country in Asia. Microscopic investigation of a urethral swab revealed Gram-negative intracellular diplococci. The patient was immediately treated empirically with ceftriaxone 500 mg single intramuscular dose plus azithromycin 1 g single oral dose. A urethral swab for culture and a first-void urine sample and a pharyngeal swab for nucleic acid amplification test (NAAT; Abbott M2000 CT/NG assay) were taken. The Asian female could not be traced. The patient had no other sexual contacts since his return from Asia; and was advised to abstain sexual intercourse until follow up visit and test of cure (TOC). The culture yielded *N. gonorrhoeae* (isolate IR72) and the NAAT on the urine sample detected *N. gonorrhoeae* DNA, but the NAAT on the pharyngeal swab was *N. gonorrhoeae* negative. A TOC was performed, using NAAT on a urine sample taken 8 days after treatment, and shown to be negative 3 days later; all signs and symptoms were resolved at this follow up visit.

**Characterisation of Neisseria gonorrhoeae isolate IR72**

Species identification of IR72 was performed using VITEK-MS (Biomerieux, Marcy l’Etoile, France) and a porA pseudogene PCR [6]. Antimicrobial susceptibility
testing was done (in duplicate) using minimum inhibitory concentration (MIC) gradient strip tests for seven antimicrobials and results were interpreted using breakpoints stated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [7]. The gonococcal reference strain ATCC 49226 was used for quality control; β-lactamase testing was performed as previously described [8].

IR72 showed resistance to ceftriaxone (MIC = 0.5 mg/L), cefixime (MIC = 1–2 mg/L), cefotaxime (MIC = 2–4 mg/L), ciprofloxacin (MIC > 32 mg/L), and intermediate resistance to azithromycin (MIC = 0.38–0.5 mg/L). IR72 was susceptible to spectinomycin (MIC = 16 mg/L) and tetracycline (MIC = 0.5 mg/L) and did not produce β-lactamase.

WGS was performed on Illumina MiSeq, as previously described [9]. The IR72 genome sequence was compared with previously genome-sequenced isolates from Ireland [10], and the ceftriaxone-resistant isolates FC428 from Japan [1], GK124 from Denmark [4], F90 from France [5], and WHO Q from the UK [11], MLST sequence types (STs), antimicrobial susceptibility to ceftriaxone, cefixime, azithromycin and ciprofloxacin, and relevant antimicrobial resistance determinants, are also shown.

The colour coding is indicated in the columns on the right.
identical to the penA allele in FC428, GK124, F90, and WHO Q [1,4,5,11]. Mosaic penA-60.001, which might originate from N. cinerea [12], encodes a mosaic penicillin-binding protein 2 (PBP2) including the key resistance-mediating amino acid substitutions A31V, I312M, V316T, T483S, and G545S [13]. IR72 additionally harboured the characteristic single nucleotide polymorphism (SNP; adenine) in the mtrR promoter inverted repeat sequence and the G120K and A121N amino acid substitutions in PorB1b, which enhance the extended-spectrum cephalosporins MICs and are associated with increased MICs of additional antimicrobials such as azithromycin, ciprofloxacin and tetracycline [13]. No 23S rRNA gene mutation associated with azithromycin resistance was found, so the intermediate azithromycin resistance was due to the mtrR resistance determinant and possibly additional unknown mutations. The S91F and D95A substitutions in GyrA (subunit A of DNA gyrase) and the S87R substitution in ParC (subunit C of Topoisomerase IV) caused the high-level resistance to ciprofloxacin [13]. The NG-STAR type of IR72 (1133) differs from the one of FC428 (type 233) by only one SNP in one (porB) of the seven NG-STAR loci. The draft genome sequence of IR72 can be found under study accession number: PRJEB29520.

The WGS phylogenomic analysis (Figure) showed that IR72 was highly different to all the previously genome-sequenced Irish isolates with decreased susceptibility or resistance to extended-spectrum cephalosporins from 2014-2016 and to WHO Q cultured in the UK in 2018 [11]. However, IR72 was belonging to the clade consisting of FC428 cultured in Japan in 2015 and the FC428 subclones identified in 2017 in Denmark (GK124 [4]) and France (F90 [5]). The whole genome of IR72 differed by 2,062 SNPs to the genome of WHO Q, but only by 60, 71, and 87 SNPs to the genomes of FC428, F90 and GK124, respectively.

Discussion and conclusions
Here, we describe the detailed characterisation of the first MDR isolate with ceftriaxone resistance and intermediate resistance to azithromycin cultured from a heterosexual male with urethritis in August 2018 in Ireland. Ceftriaxone resistance in N. gonorrhoeae remains rare internationally [14-16]. Phenotypic and WGS characterisation showed that the Irish isolate described here belongs to the ceftriaxone-resistant and MDR FC428 clone initially described in Japan in 2015 [5]. Minor genomic changes were identified, which likely represent the evolution of FC428.

FC428 subclones were reported in 2017 in Australia, Canada, Denmark, and France [2-5] and are the first evidence of a ceftriaxone-resistant gonococcal clone that appears to have maintained a high fitness and spread internationally. Detailed examination of the phenotypic and genetic characteristics, including the fitness of the FC428 clone and its evolving subclones, is therefore imperative. Furthermore, enhanced surveillance of gonococcal antimicrobial resistance and gonorrhoea treatment failures is needed, particularly in the South-East Asian and Western Pacific Region, where FC428 and many of the FC428-associated descendants [1-5] have originated from. Most worryingly, no sexual partner(s) of the index patient in the present paper and in previous instances of infections with FC428 or its subclones [1-5] could be traced in Asia. Notably, the first gonococcal strain with ceftriaxone resistance plus high-level azithromycin resistance (WHO Q) was identified in England in 2018 [11], followed by two similar cases in Australia [17]. Two of these three cases were also associated with travel to South-East Asia [11,17].

Awareness of the international spread of FC428, its subclones and additional ceftriaxone-resistant strains that are threatening recommended dual therapies (ceftriaxone plus azithromycin) needs to be enhanced. In addition, surveillance of antimicrobial resistance and treatment failures (ideally supplemented by WGS), improved implementation of dual antimicrobial therapies with high dose of ceftriaxone and azithromycin [18], successful notification and treatment of sexual partners and TOC are essential on an international level. Further, new antimicrobials for treatment of gonorrhoea and ideally an effective gonococcal vaccine, as a long-term solution for management and control of gonorrhoea, are essential.

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Conflict of interest
None declared.

Authors’ contributions
BC and MU designed and initiated the study; AL was involved in managing the clinical case; DG, BC, LR, AM and MU coordinated and performed all the laboratory experiments; BB and MO shared genomic sequences from previous cases; DG, BC and MU analysed all the genomic data and wrote a first draft of the paper. All authors were involved in finishing the final version of the paper.

References


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Acute flaccid paralysis (AFP) surveillance is key for global polio eradication. It allows detecting poliovirus (PV) reintroductions from endemic countries. This study describes AFP surveillance in Spain from 1998 to 2015. During this time, 678 AFP cases were reported to the Spanish National Surveillance Network. The mean notification rate was 0.58 AFP cases/100,000 population under 15 years old (range: 0.45/100,000–0.78/100,000). Two periods (P) are described: P1 (1998–2006) with the AFP notification rate ranging from 0.66/100,000 to 0.78/100,000, peaking in 2001 (0.84/100,000); and P2 (2007–2015) when the AFP rate ranged from 0.43/100,000 to 0.57/100,000, with the lowest rate in 2009 (0.31/100,000). No poliomyelitis cases were caused by wild PV infections, although two Sabin-like PVs and one imported vaccine-derived PV-2 were detected. Overall, 23 (3.4%) cases met the hot case definition. Most cases were clinically diagnosed with Guillain–Barré syndrome (76.9%; 504/655). The adequate stool collection rate ranged from 33.3% to 72.5% (29/40). The annual proportion of AFP cases with non-polio enterovirus findings varied widely across the study period. AFP surveillance with laboratory testing for non-polio enteroviruses must be maintained and enhanced both to monitor polio eradication and to establish sensitive surveillance for.
Introduction

The 1988 Global Polio Eradication Initiative (GPEI) included four strategies: (i) high systematic immunisation with oral polio vaccine (OPV); (ii) provision of supplementary vaccinations; (iii) active surveillance of wild poliovirus (WPV) through reporting and laboratory testing of all acute flaccid paralysis (AFP) cases in children <15 years of age; (iv) once transmission has been limited, development of campaigns to reach any unvaccinated population [1].

By 2011, four of the six World Health Organization (WHO) Regions had seen their last indigenous polio cases and were certified as polio free. But polio proved to be insidious and poliovirus transmission has persisted in countries with ineffective immunisation systems and low-performing eradication activities, resulting in potential risk of international spread and virus importations into previously polio-free countries [2].

Despite the dramatic decrease in worldwide polio cases in the last few decades, the WHO estimates that, from 2003 to 2014, there were 191 importation events into previously polio-free countries, resulting in 3,763 reported cases of paralytic polio in 43 countries. A Public Health Emergency of International Concern was declared by the WHO in 2014 [3]. Since then the International Health Regulations Committee recommends that countries in which WPV or vaccine-derived poliovirus (VDPV) transmission is occurring should ensure and document that residents and long-term visitors (i.e. > four weeks) receive a dose of polio vaccine.

Figure 1
Cases of poliomyelitis, type of polio vaccines and vaccination coverage, Spain, 1931–2015

IPV: inactivated polio vaccine; OPV: oral polio vaccine.

Vaccination coverage with three doses of polio vaccine by 12 months of age.

Source: National Centre of Epidemiology, Instituto de Salud Carlos III and Ministry of Health.

**prompt detection of other enteroviruses causing serious symptoms.**
prior to international travel. Restriction of international travel of any resident lacking documentation of appropriate polio vaccination should be imposed at the point of departure [3].

On rare occasions, vaccine-associated paralytic poliomyelitis (VAPP) may occur up to 30 days after receiving an OPV dose. Sabin polioviruses can also replicate in immunocompromised persons (iVDPV) acquiring the neurovirulence and transmissibility characteristics of WPV and they can spread in populations with low OPV coverage causing circulating VDPV (cVDPV) cases and outbreaks [4].

In September 2015, worldwide eradication of indigenous WPV type-2 was declared. To stop circulation of VDPV type-2, live attenuated type-2 poliovirus was withdrawn from oral vaccines as at April 2016. Nevertheless outbreaks caused by cVDPVs, including cVDPV type-2, have continued in areas of several countries [5]. This implies that long-term use of OPV poses an ongoing risk and, consequently the GPEI will need to stop OPV vaccination in addition to certification and WPV containment [5].

The European Regional Commission for Poliomyelitis Eradication (RCC) publishes an annual report with the main features of national reports and conclusions about the polio-free status of the Region. The possibility of re-entry of the virus into Europe can never be completely discarded primarily because of low population immunity in some countries [6].

The National Certification Committee (NCC) for polio eradication was established in Spain in 1998 as a component of the National Plan of Actions Aimed at the Achievement of the Certificate of Polio Eradication (NPCPE) [7]. An annual update on polio eradication activities is prepared by the NCC and submitted to the RCC. The report contains a statement presenting the evidence-based rationale for the absence of poliovirus circulation in the country.

Updated versions of the NPCPE were approved in Spain in 2007, 2011 and 2016. Nationwide, the main
Poliovirus eradication activities are: maintenance of high vaccination coverage and the strengthening of poliovirus surveillance, including AFP and enterovirus (EV) surveillance [7-9].

Since the implementation of the NPCPE and the development of the AFP surveillance system, the sensitivity of the surveillance has gradually decreased in relation to the WHO objective of being able to detect at least one case of AFP per 100,000 population under 15 years old [9]. In Spain, OPV was replaced by inactivated polio vaccine (IPV) in 2004. Since 1996, national coverage of three doses in children’s first year of age exceeds 95%. In 2015, national coverage was 96.4% (ranging between 92.8 and 100.0% according to autonomous regions) (Figure 1).

The objective of this study is to report and evaluate the results of 18 years (1998–2015) of the surveillance programme for AFP in Spain in terms of incidence, epidemiological and laboratory investigations, clinical diagnosis, as well as utility and quality of surveillance.

### Methods

**Acute flaccid paralysis surveillance system in Spain**

In Spain, AFP surveillance is the main component of poliovirus surveillance, along with supplementary EV laboratory surveillance. Environmental surveillance is not established nationwide. Laboratory techniques and protocols that should be maintained or could be developed in the event of PV importation or an outbreak are assessed annually by a pilot environmental study in an urban area. The results are reported yearly to the RCC.

AFP surveillance is mandatory in Spain. It is conducted by the Spanish Epidemiological Surveillance System (RENAVE) together with the AFP Surveillance Laboratory Network, and is coordinated by the National Epidemiology Centre (CNE) of the Carlos III Health Institute (ISCIII). The Regional Epidemiological Surveillance Centres (RESCs) are located in each of the 19 autonomous territories (17 autonomous regions and two autonomous cities). These surveillance centres are
made up of local networks of public and private hospitals (paediatric, neurology and infectious disease wards) which are directly coordinated by the person in charge of the RESCs (Figure 2).

Case notification as well as other relevant information is collated centrally and subsequently fed-back through the system, between the different levels: local, regional, national and international (Figure 2).

Laboratory network

The AFP Surveillance Laboratory Network coordinated by the National Poliovirus Laboratory (NPL) of the ISCIII was set up in 1998. The NPL is part of the European Polio Laboratory Network and is accredited annually by the WHO. In Spain the number of regional laboratories belonging to the AFP surveillance network has been reduced over time, decreasing from nine in 1998 to only three in 2015. The network laboratories perform virological studies of notified AFP cases in their region, while the national laboratory studies all AFP cases reported by the 16 autonomous territories without regional laboratories.

The network laboratories perform virological studies of stool samples using cell culture techniques – following protocols recommended by the WHO [10,11]. Since 2015 a new diagnosis policy has been introduced in all European polio laboratories and now culture results must be available within 14 days after laboratories receive samples, instead of 28 days earlier [9]. Laboratory analysis consists of the evaluation of viral growth after two blind passages on cell lines (RD and L20B) sensitive to poliovirus infection (3 serotypes) and to most of the non-polio EV (NPEV) infections. Negative results refer to the absence of viral growth. Any positive result must be sent to the national laboratory immediately. The NPL is responsible for confirming EV detection and characterising the serotype. Any virological results will be notified to the WHO through the Laboratory Data Management System (LDMS) (Figure 2).

Reporting and investigation of acute flaccid paralysis cases

The RESCs must notify the CNE of any AFP cases occurring in the 0–14 year-old population. In the event of an AFP case, a preliminary reporting form with clinical and epidemiological information has to be completed [12]. A hot case is a person highly suspected for polio based on the clinical suspicion of poliomyelitis, and who has either received less than three doses of polio vaccine, or has recently travelled to an endemic country (having returned up to 35 days before the onset of paralysis) or belongs to a high risk group [13]. If an AFP hot case is reported, a set of Standard Operating Procedures (SOP), including priority for laboratory investigation and contact tracing, must be implemented [9] (Figure 3).

Clinical specimens for virological investigation have to be collected. A follow-up reporting form is sent to CNE to determine paralysis evolution after 60 days from paralysis onset, and to clarify the final diagnosis of the case. Cases are classified as confirmed, discarded or compatible with polio [12,13] (Figure 3).

Zero reporting

To monitor AFP surveillance quality zero reporting is recommended. The collaborating hospitals in the region should respond to RESC enquiries through a contact-point among the medical staff, who acts as a link between clinicians and epidemiologists for surveillance activities. To maintain the highest coverage, a number of hospitals report for each region: Andalucía (n = 33); Aragón (n = 9); Asturias (n = 1); Baleares (n = 14); Cantabria (n = 1); Canarias (n = 4); Castilla La Mancha (n = 12); Castilla y León (n = 18); Cataluña (n = 14); Comunidad Valenciana (n = 28); Extremadura (n = 8); Galicia (n = 10); Madrid (n = 18); Murcia (n = 10); Navarra (n = 1); País Vasco (n = 6); La Rioja (n = 2); Ceuta (n = 1) and Melilla (n = 1). On a monthly basis, medical
### Table 1a

Acute flaccid paralysis (AFP) surveillance system, AFP cases expected and reported, and quality indicators of surveillance performance, Spain, 1998–2006

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description of the parameters</th>
<th>WHO target</th>
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<td><strong>Detection and investigation</strong></td>
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<td></td>
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<tr>
<td>AFP cases reported</td>
<td>No further description</td>
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<td>48</td>
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<td>37</td>
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<td>Cases expected = Total population under 15 years x (1/100,000)</td>
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<td>59</td>
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<td>64</td>
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<tr>
<td>Population &lt; 15 years</td>
<td>No further description</td>
<td>NA</td>
<td>5,897,436</td>
<td>5,882,353</td>
<td>5,925,926</td>
<td>6,071,429</td>
<td>6,065,574</td>
<td>6,081,081</td>
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<td>6,027,397</td>
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<tr>
<td>AFP detection rate</td>
<td>Number of AFP cases x 100,000/total population under 15 years</td>
<td>&gt; 1</td>
<td>0.78</td>
<td>0.68</td>
<td>0.81</td>
<td>0.84</td>
<td>0.61</td>
<td>0.74</td>
<td>0.72</td>
<td>0.73</td>
<td>0.66</td>
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<tr>
<td>Reporting rate</td>
<td>% AFP cases with delay between paralysis onset and notification ≤ 7 days</td>
<td>≥ 80%</td>
<td>ND</td>
<td>55.0%</td>
<td>50.0%</td>
<td>45.0%</td>
<td>48.0%</td>
<td>43.0%</td>
<td>35.0%</td>
<td>50.0%</td>
<td>40.5%</td>
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<td>Investigation rate</td>
<td>% AFP cases with delay between notification and investigations ≤ 2 days</td>
<td>≥ 80%</td>
<td>97.0%</td>
<td>97.5%</td>
<td>98.0%</td>
<td>96.0%</td>
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<td>100%</td>
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<td>100%</td>
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<tr>
<td>Stool collection rate</td>
<td>% AFP with one faecal specimen within 14 days of paralysis onset</td>
<td>≥ 80%</td>
<td>91.3%</td>
<td>92.5%</td>
<td>97.9%</td>
<td>100%</td>
<td>94.6%</td>
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<td>100%</td>
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<td>95.2%</td>
</tr>
<tr>
<td>Adequate stool collection rate</td>
<td>% AFP with two faecal specimens taken ≥ 1 day apart within 14 days of paralysis onset</td>
<td>≥ 80%</td>
<td>64.0%</td>
<td>73.0%</td>
<td>69.0%</td>
<td>61.0%</td>
<td>57.0%</td>
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<td>56.0%</td>
<td>65.0%</td>
<td>45.2%</td>
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<tr>
<td>Surveillance index</td>
<td>Surveillance index = AFP detection rate up to 1.0 x adequate stool collection rate</td>
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<td>0.50</td>
<td>0.56</td>
<td>0.51</td>
<td>0.35</td>
<td>0.34</td>
<td>0.50</td>
<td>0.48</td>
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<tr>
<td>Zero-reporting completeness</td>
<td>Annual number of AFP zero-reporting forms submitted/number of zero-reporting forms expected in the reporting year x 100</td>
<td>≥ 80%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>97.4%</td>
<td>95.6%</td>
<td>ND</td>
<td>99.8%</td>
<td>96.5%</td>
<td>98.4%</td>
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<tr>
<td>Zero-reporting timeliness</td>
<td>Annual number of AFP zero-reporting forms submitted by the first 7 days of the following month/number of forms expected in the reporting year x 100</td>
<td>≥ 80%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>73.8%</td>
<td>68.6%</td>
<td>ND</td>
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<td>43.9%</td>
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<tr>
<td>Hot cases</td>
<td>Case with a priority code (less than three doses of polio vaccine/clinical presentation compatible with polio/recent travel to endemic country/high risk group)</td>
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<td>2</td>
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<td>2</td>
<td>6</td>
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<td>2</td>
<td>1</td>
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<td>% AFP cases with follow-up 60 days after the date of paralysis onset</td>
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<td>94.2%</td>
<td>92.0%</td>
<td>96.0%</td>
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<td>Adequate laboratory results rate</td>
<td>% AFP cases with laboratory results ≤ 28 days of receiving samples at laboratory</td>
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<td>81.4%</td>
<td>90.0%</td>
<td>68.0%</td>
<td>82.0%</td>
<td>70.0%</td>
<td>90.0%</td>
<td>81.0%</td>
<td>82.1%</td>
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<tr>
<td></td>
<td></td>
<td>≥ 80% (2nd)</td>
<td>81.1%</td>
<td>97.0%</td>
<td>67.0%</td>
<td>89.0%</td>
<td>90.0%</td>
<td>90.0%</td>
<td>86.0%</td>
<td>81.3%</td>
<td>93.8%</td>
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<tr>
<td>Non-polio enterovirus typing (NPEV) (%)</td>
<td>% AFP cases with positive non-polio enterovirus finding</td>
<td>≥ 10%</td>
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<td>8.1%</td>
<td>2.1%</td>
<td>9.8%</td>
<td>11.4%</td>
<td>5.0%</td>
<td>4.5%</td>
<td>2.6%</td>
<td>10.0%</td>
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</table>

AFP: acute flaccid paralysis; NA: not applicable; ND: not determined; WHO: World Health Organization.

*a* Since 2015: ≤ 14 days from receiving samples at laboratory is accepted.

Source: National Centre of Epidemiology. Instituto de Salud Carlos III.
### Acute Flaccid Paralysis (AFP) Surveillance System, AFP Cases Expected and Reported, and Quality Indicators of Surveillance Performance, Spain, 2007–2015

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<tr>
<td>Stool collection rate</td>
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<tr>
<td>Adequate stool collection rate</td>
<td>Adequate stool collection rate</td>
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<tr>
<td>Priority cases and follow-up</td>
<td>Priority cases and follow-up</td>
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<td>100%</td>
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<td>100%</td>
<td>95.7%</td>
<td>100%</td>
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</tr>
<tr>
<td>Follow-up AFP rate</td>
<td>Follow-up AFP rate</td>
<td>97.8%</td>
<td>95.6%</td>
<td>94.3%</td>
<td>97.3%</td>
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<tr>
<td>Zero-reporting completeness</td>
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<td>94.3%</td>
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<tr>
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<td>Zero-reporting timeliness</td>
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<td>95.6%</td>
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<tr>
<td>Laboratory results</td>
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<td>95.6%</td>
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<tr>
<td>Non-polio enterovirus (NPEV) (%)</td>
<td>Non-polio enterovirus (NPEV) (%)</td>
<td>2.9%</td>
<td>9.7%</td>
<td>0.0%</td>
<td>9.5%</td>
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<td>9.5%</td>
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</tr>
</tbody>
</table>

AFP: acute flaccid paralysis; NA: not applicable; WHO: World Health Organization.

Since 2015-2016: <14 days from receiving samples at laboratory is accepted.

Source: National Centre for Epidemiology, Instituto de Salud Carlos III.
staff are asked to report non-notified AFP cases in hospitalised children. If AFP cases are not found, a zero-reporting form should be sent to the CNE in the first 7 days of the following month. The completeness and the timeliness of zero reporting are used as surveillance quality indicators (see Table 1 in the results’ section).

Assessing acute flaccid paralysis surveillance by reviewing hospital discharge registries
Retrospective searches of medical records which meet AFP clinical criteria help to determine a baseline burden for AFP in a country. Annually, each RESC conducts a retrospective search at the Regional Hospitalization Discharge Registry. The results of these searches are reported to the CNE. The cause of hospitalisation is considered to be clinically compatible with AFP when the Ninth International Classification of Diseases (ICD-9-CM) [14] codes are 357.0 and 356.9 (Guillain–Barré Syndrome or other peripheral neuropathy), 336.9 (acute myelitis) or 045.0 and 045.1 (acute poliomyelitis). The information on follow-up was collected in 651 cases (96.0%), of which 179 (27.4%) presented residual paralysis after 60 days of paralysis onset. Among them, 23 (3.4%) met hot case definition, including two VAPPs and another paralysis case produced by an iVDPV (Table 1). The risk factors associated were: clinical suspicion of poliomyelitis (3 cases), having had less than three doses of polio-vaccine (16 cases) and being immunosuppressed (9 cases); six cases had more than one risk factor concurrently.

Assessment of quality of acute flaccid paralysis surveillance performance
A set of indicators are calculated annually at national level and compared with WHO standards. Indicators evaluate the main system attributes: sensitivity, completeness and timeliness of notification, investigation and laboratory workup. To synthesise the quality of AFP surveillance WHO requests the ‘surveillance index’ (see Table 1 in the results’ section).

Data analysis
Individualised information regarding notified AFP cases between 1998 and 2015 in Spain was obtained from the RENAVE database. Zero-case monthly reports and data from hospital discharge registries between 2000 and 2015 were obtained from the AFP surveillance system. To calculate the AFP notification rate, population data were obtained from the National Institute for Statistics.

Results
Acute flaccid paralysis detection and demographic variables
A total of 678 AFP cases were notified between 1998 and 2015. The annual number of AFP cases reported ranged from 51 (in 2001) to 21 (in 2009) respectively. All of the cases were discarded for polio except two VAPPs (1999; 2003) and one paralysis case associated with an iVDPV (2005). The AFP detection rate in the population under 15 years decreased over the study period, from 0.78 AFP cases/100,000 in 1998 to 0.45/100,000 in 2015; the mean AFP detection rate was 0.58/100,000. Two periods (P) are described regarding AFP detection. The first, P1 (1998–2006), was when the AFP rate ranged from 0.78/100,000 in 1998 to 0.66/100,000 in 2006; the rate peaked in 2001 at 0.84/100,000. The second period, P2 (2007–2015), showed an AFP rate ranging from 0.57/100,000 in 2007 to 0.43/100,000 in 2015. The lowest ever reported rate was 0.31/100,000 in 2009 (Table 1; Figure 4).

By age group, most cases (310, 45.7%) were under 5 years old; 29.5% (200) were between 5 and 9 years old and 24.8% (168) were between 10 and 14 years old; the youngest case was a newborn aged 16 days. Overall, a slightly higher proportion of male cases were reported (383/678, 56.4%).

Vaccination status was recorded for 659 cases (97.2%). Among them, 637 cases (96.7%) had received three or more doses of polio vaccine before being diagnosed with AFP; two cases (0.3%), both in the 5–9 year age group, were completely unvaccinated; and a further 20 cases (3.0%) had received less than three doses, including 16 under-vaccinated cases older than 6 months.

Of all AFP cases, 23 (3.4%) met hot case definition, including two VAPPs and another paralysis case produced by an iVDPV (Table 1). The risk factors associated were: clinical suspicion of poliomyelitis (3 cases), having had less than three doses of polio-vaccine (16 cases) and being immunosuppressed (9 cases); six cases had more than one risk factor concurrently.

Clinical features
Key clinical signs suggestive of polio were collected. For cases with available information, limbs were the most common site of paralysis (535/610, 87.7%); 20.2% (130/642) had fever (≥38°C) at paralysis onset; 40.4% (227/562) presented rapid progression to complete paralysis and 8.2% (50/610) had asymmetric paralysis. All the AFP cases were hospitalised, mainly in highly specialised tertiary hospitals. For 655 (96.6%) of them a clinical diagnosis was reached. Among them, Guillain–Barré syndrome (GBS), including Miller Fisher syndrome and other polyradiculoneuritis, was the most common clinical diagnosis in each year of the whole study period (504, 76.9%), followed by myelitis (44 cases, 6.7%) and other uncommon diagnoses like encephalomyelitis or meningitis (23 cases, 3.5%). Other rare reported diagnoses were tumours or multiple sclerosis.

The information on follow-up was collected in 651 cases (96.0%), of which 179 (27.4%) presented residual paralysis after 60 days of paralysis onset.

Most cases were classified as non-polio AFP (675, 99.0%), except three cases who occurred due to a Sabin-like poliovirus (PV-SL) or an iVDPV. In 1999 a 5-month-old infant presented with rapidly evolving asymmetric paralysis, having received the first OPV dose one month before; a PV-SL3 was detected in stool samples. The infant recovered but residual paralysis persisted. In 2001, a 7-month-old infant, having received the second dose of OPV 3 months before, presented with asymmetric paralysis with a rapid evolution and PV-SL2 was identified in stool samples.
## Table 2
Poliovirus and non-polio enterovirus detected in stool samples from reported acute flaccid paralysis cases, Spain, 1998–2015

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AFP: acute flaccid paralysis; EV: enterovirus; iVDPV: immunocompromised vaccine-derived poliovirus; NPEV: non-polio enterovirus; PV: poliovirus; PV-SL: Sabin-like poliovirus; VAPP: vaccine-associated paralytic poliomyelitis.

\textsuperscript{a}1 VAPP-PVSL\textsuperscript{3}.

\textsuperscript{b}1 VAPP-PVSL\textsuperscript{2}.

\textsuperscript{c}Imported case.

Source: National Poliovirus Laboratory and National Centre of Epidemiology, Instituto de Salud Carlos III.
Concurrent severe immunosuppression was diagnosed and the child died.

Later, in 2005, an AFP case imported from Morocco and resulting from a recombinant iVDPV type 2 was found. The case was a 14-month-old boy with residual paralysis and major histocompatibility complex Class II immunodeficiency; he had received two OPV doses, the second at 6 months of age. Eight months later the patient developed encephalitis with clinical tetraparexia, at which point suspicion of polio was raised and diagnosis confirmed. The high level of vaccination coverage in Spain and the rapid tracing and monitoring of close contacts prevented virus spread [15].

**Laboratory investigations**

For 92.3% (626) of the total AFP reported cases, at least one stool sample was collected. Taking into account that several samples from the same person could be collected, from 1998 to 2004, 14 stool samples taken from AFP cases or from their contacts were positive for PV-SL. A PV-SL3 and a PV-SL2 were isolated from two VAPP cases. In addition, during 2005, an iVDPV type 2 was detected (Table 2). Furthermore, the NPL performs the characterisation of all NPEVs isolated. EV species B were the most frequent serotypes found in the stool samples (30; 4.4% of AFP cases), although several Coxsackievirus A (from species A) were also detected. Samples (30; 4.4% of AFP cases) were collected, from 1998 to 2004, 14 stool samples taken from AFP cases or from their contacts were positive for PV-SL. A PV-SL3 and a PV-SL2 were isolated from two VAPP cases. In addition, during 2005, an iVDPV type 2 was detected (Table 2). Furthermore, the NPL performs the characterisation of all NPEVs isolated. EV species B were the most frequent serotypes found in the stool samples (30; 4.4% of AFP cases), although several Coxsackievirus A (from species A) were also detected. An EV-D68 was identified in stools from a 4 year-old child with severe limb paralysis in 2015 (Table 2).

**Quality indicators of acute flaccid paralysis surveillance performance**

The quality of AFP surveillance has weakened over the surveillance system’s lifespan. The sensitivity of the surveillance, the AFP detection rate, has gradually decreased from the WHO-stated objective of at least one case per 100,000 inhabitants under 15 years of age (Table 1; Figure 4). The completeness of zero reporting has always been maintained at over 80%, while the timeliness of zero reporting decreased from 73.8% in 2001 to 34.2% in 2012 (Table 1). The number of hospitalisations with an AFP diagnosis identified in hospital discharge registry searches shows a fluctuating pattern. A variable gap is described between the AFP hospitalisation rate and the AFP detection rate across the study period (Figure 4).

Timeliness of investigation (i.e. the investigation rate) reached the 80% target every year and for most cases at least one faecal specimen was taken; however, the ‘adequate stool collection rate’ always remained under the expected 80%, and seemed to decrease slightly during the second period (2007–2015) compared with the first period (1998–2006). Consequently, the ‘surveillance index’ experienced a decrease from 0.50 in 1998 to 0.21 in 2015 (Table 1).

The ‘adequate laboratory results rate’ has been always above 80% except for 2015, when the new diagnosis policy began and the laboratory results had to be communicated sooner (14 days instead of 28 days). After two-year period of adaptation, laboratory capabilities recovered; for 2017 the ‘adequate laboratory results rate’ exceeded the quality target required (82.4% for the first sample and 85.7% for the second one) [16]. During the study period, the proportion of AFP cases with positive NPEV findings ranged from 17.2% in 2010 to 0% in 2009 and 2014 (Table 1).

**Discussion**

In Spain the last endemic case produced by a WPV occurred in 1988 in an under-immunised population. After that event Spain progressively improved the polio vaccination coverage (since 1996 national coverage of three doses exceeds 95%) allowing to reach a high population immunity against polio. Three additional imported cases associated with WPV were reported between 1980 and 1989. Since 2004 OPV is no longer administered in Spain and the last cases associated with PV-SL were reported in 1999 and 2001 [8,17-19]. The European Regional Certification Commission for Poliomyelitis Eradication classifies Spain as at low risk of WPV transmission. The maintenance of high polio vaccination coverage minimises the risk of spread in case of importation, and a good-quality surveillance system ensures the timely poliovirus detection [6].

The quality of AFP surveillance decreased with time, particularly after the OPV to IPV switch, reflecting the loss of awareness about polio, when finding cases of paralysis produced by WPV or associated to vaccine virus are very unlikely.

During the studied period, the number of AFP cases reported underwent similar fluctuations to the rate of children hospitalised with a clinical compatible AFP diagnosis; the fact that the hospitalisations and notifications follow the same pattern, indicates that the surveillance is working relatively well despite it detecting less than one case of AFP per 100,000.

In our experience, the WHO’s expected rate of at least one case of AFP per 100,000 population under 15 years of age could be overestimating the real rate of this syndrome among children in a country like Spain.

Although active feedback has been proven not to increase the proportion of reported and virologically investigated patients [20], in our experience zero reporting acts as a monthly reminder maintaining awareness about polio among clinicians who tend to believe it is a long-disappeared disease. It also ensures that the entire country takes part in the surveillance and the whole population is represented. In Spain, zero cases are reported, but the delay in notification is increasingly evident.

About half of reported AFP cases are notified more than a week after paralysis onset. Low concern levels regarding polio and the delay in consulting for symptoms of insidious onset, such as some type of paralysis,
are the main causes of this reporting delay and may minimise the chance to implement control measures and/or appropriate sample collection. This is reflected by the ‘adequate stool collection rate’, which did not reach the expected 80% over the study period and the decrease of the ‘surveillance index’ during that time. Nevertheless, the investigation rate every year reaches the 80% target, showing that once a case enters the system, the investigation is conducted promptly.

In addition to discarding poliovirus infection, AFP laboratory diagnosis can lead to identifying other EVs in patients’ stool samples. In fact, the proportion of AFP cases with positive NPEV findings is used as an indicator for the quality of the laboratory’s EV typing.

The most frequent EVs identified in the samples from AFP cases across the study period were EVs from species B (echovirus 3, echovirus 7 and echovirus 30); similar results were found in laboratory EV surveillance [17]. A systematic review about EV detections in cases with AFP showed that in surveillance studies, EVs from species B were among the most frequently detected [21].

The recent increase in the detection of EVs associated with severe neurological symptoms in children across Europe [22,23] has also been identified in Spain. Of particular concern was an EV-A71 outbreak notified in 2016 with many severe encephalitis cases in paediatric patients [24,25].

The first case of paediatric neurological disease associated to EV-D68 infection in Spain was reported to the national AFP surveillance system late in 2015 [16]. During 2016, two additional AFP cases associated with EV-D68 [26] and 12 AFP cases associated with EV-A71 were reported highlighting the importance of maintaining AFP surveillance to detect concerning signals of unexpected EV circulation in a country [16]. Any surveillance system devoted to monitoring the spread of EVs should take advantage of existing networks, such as AFP surveillance systems, non-polio EV laboratory networks or viral meningitis surveillance [22,27,28].

In a polio-free IPV-user country, poliomyelitis can arise and spread to contacts who are not properly vaccinated. Movements of people from areas still using OPV, under-vaccinated population groups, foreign-born children coming from endemic countries or hard-to-reach communities can lead to the identification of AFP hot cases, and will trigger the Standard Operational Procedure until the case can be discarded [9]. The aim of the response plan is to implement activities aimed at interrupting any poliovirus transmission within 120 days of confirmation. The response activities will be promptly implemented when a suspected case of polio or priority AFP case is reported, or when a poliovirus is identified by the EV surveillance or by the environmental surveillance system [9].

Re-emergence still may arise from prolonged asymptomatic excretion of poliovirus by hospitalised primary immune deficient (PID) patients, through repeated exposure of close contacts. PID patients with a recent OPV vaccination should be identified and screened for any poliovirus excretions [29].

In 2017 no WPV transmission in the WHO European Region occurred [6]. One of the milestones achieved in 2016 was the successful switch from trivalent to bivalent polio vaccine by all countries in the Region that still used OPV [30]. Cessation of the use of OPV is necessary to eliminate the low long-term risks of VDPVs associated with its use. Countries with IPV in their vaccination schedule commonly share borders with OPV-user countries, and residents may travel back and forth, increasing the risk of VDPVs’ circulation.

The quality of AFP surveillance in the WHO European Region as a whole has not declined in recent years, but there are indications that vaccine coverage is in decline in a small number of countries, which is of concern. In particular, Bosnia and Herzegovina, Romania and Ukraine were considered to be at high risk of a sustained polio outbreak due to suboptimal performance of poliovirus surveillance and the low population immunity [6]. A further 25 countries have been considered as having an intermediate risk and an additional 24 as having a low risk of sustained transmission in the event of WPV importation or emergence of VDPV [6].

Surveillance of AFP is considered as a milestone and the most efficient form of surveillance in the last phase of polio eradication. Maintaining clinical surveillance of poliomyelitis in a polio-free territory is hard. Poliomyelitis is among 67 other diseases under mandatory notification to the Spanish National Surveillance System. Reviewing the history of AFP surveillance in Spain between 1998 and 2015 brings up the need for awareness by all healthcare professionals involved: medical staff in charge of paediatrics, child neurology or paediatric intensive care units and the personnel from the laboratories involved in diagnosis of these patients’ clinical samples.

AFP surveillance along with non-polio EV laboratory surveillance must be maintained and enhanced both to help polio eradication and to establish a sensitive surveillance for prompt detection of emergent and unexpected circulation of other EVs.

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Conflict of interest

None declared.

Authors’ contributions

JMC, NT and NLP were the main study researchers. They planned the content and wrote the first version of the manuscript. JMC performed data collection and statistical analysis. NLP, NT and BFM contributed to the analysis of epidemiological data and to performing tables and graphs; MC collected laboratory data. All of them made a critical review of all versions of the manuscript. NT, VGG, CM, MM, MP, NA, AB, SGH, HM, NR, CM, AF, IL, JGG, AN,VGO, MGc, JMA, ABM, AR, DC and other members of Spanish AFP Surveillance Working Group were the epidemiologists responsible of AFP surveillance in each Regional Epidemiological Surveillance Centers (RESC). NR, SS, JMN, MO, CPG and MdC were responsible of the Spanish AFP Surveillance Laboratory Network.

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Arbovirus surveillance: first dengue virus detection in local Aedes albopictus mosquitoes in Europe, Catalonia, Spain, 2015

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Dengue has emerged as the most important viral mosquito-borne disease globally. The current risk of dengue outbreaks in Europe appeared with the introduction of the vector Aedes albopictus mosquito in Mediterranean countries. Considering the increasing frequency of dengue epidemics worldwide and the movement of viraemic hosts, it is expected that new autochthonous cases will occur in the future in Europe. Arbovirus surveillance started in Catalonia in 2015 to monitor imported cases and detect possible local arboviral transmission. During 2015, 131 patients with a recent travel history to endemic countries were tested for dengue virus (DENV) and 65 dengue cases were detected. Twenty-eight patients with a febrile illness were viraemic, as demonstrated by a positive real-time RT-PCR test for DENV in serum samples. Entomological investigations around the viraemic cases led to the detection of DENV in a pool of local Aedes albopictus mosquitoes captured in the residency of one case. The sequence of the DENV envelope gene detected in the mosquito pool was identical to that detected in the patient. Our results show how entomological surveillance conducted around viraemic travellers can be effective for early detection of DENV in mosquitoes and thus might help to prevent possible autochthonous transmission.

Introduction

The rapid geographical spread of invasive mosquitoes and vector borne diseases (VBD) as well as their increasing burden are global concerns [1]. Dengue has emerged as the most important viral mosquito-borne disease globally. In a study from 2013, Bhatt et al. [2] estimated that there were 390 million human dengue infections per year worldwide, including 96 million with clinical manifestations. Dengue is caused by dengue virus (DENV), which has four different serotypes (DENV-1 to DENV-4), each of which is further subdivided into distinct genotypes [3].

DENV is transmitted through the bites of infected Aedes mosquitoes [3] and is present in tropical and subtropical regions of the world. The disease is endemic in more than 100 countries in south-east Asia, the Americas, the western Pacific, Africa and the Eastern Mediterranean. Demographic- and anthropogenic-driven environmental changes combined with globalisation and inefficient public health measures are considered the principal driving forces for the emergence and global spread of dengue in the past 40 years [4].

At the beginning of the last century, dengue had disappeared from Europe [5]. The disease had been present
in Spain at least from 1778 and probably until 1927 [5] and the last European outbreak had occurred in Greece in the years 1927–28. During this epidemic, DENV had been transmitted by *Ae. aegypti* and had caused 1 million cases with more than 1,000 deaths [6]. Following the elimination of *Ae. aegypti* in the first half of the 20th century in Europe [7], the risk of dengue mainly re-emerged with the introduction of *Ae. albopictus* in certain areas [8]. Nevertheless, it is noteworthy that on the island of Madeira *Ae. aegypti* presence has been known since 2005 and autochthonous occurrence of dengue was reported there in 2012 [9,10].

*Ae. albopictus* is an Asian mosquito species, which is a vector of several arboviruses including DENV and chikungunya virus (CHIKV). Through the global trade of used tyres and other goods, it began to expand worldwide in the 1970s [11]. From this period, it started to spread to parts of Europe, particularly near the Mediterranean Sea [8]. Travellers, who are infected with DENV or CHIKV in endemic areas of the world, and who return to European areas colonised by *Ae. albopictus*, increase the risk of introduction of the respective diseases, particularly if they are in the viraemic phase. Concerns about such a risk arose with chikungunya outbreaks in Italy in 2007 [12].

Subsequently, in the late summer of 2010, an autochthonous dengue case was reported in Nice, France. The likely index case was described as a man who lived in the proximity of the first case, and who had recently returned from Martinique where he contracted DENV infection [13]. Also in August 2010, a 72 year-old man in Germany was diagnosed with DENV infection following his return from a holiday in Croatia [14]. Because shortly after, another DENV infection was detected in the same Croatian village than the one visited by the German tourist, an autochthonous DENV circulation was suspected in that area of Croatia, prompting local investigations. Based on molecular analysis, the virus was probably introduced by a person who arrived in Croatia from the Indian subcontinent in 2010 [15]. Further autochthonous occurrences of dengue in Europe occurred in France in 2013 [16] and in 2015 [17] with probable index cases originating from Guadeloupe and French Polynesia respectively.

Considering the increasing frequency of dengue epidemics worldwide and the movement of viraemic hosts, it is expected that new autochthonous cases will continue to occur in the future in Europe where the vectors are present.

In Catalonia (north-eastern Spain), *Ae. albopictus* was first detected in 2004, corresponding to what is considered the first introduction of this species on the Iberian Peninsula and in Spain [18]. Because diagnosed imported cases of arboviral diseases increased in Catalonia from 2009 to 2013 [19], an epidemiological surveillance for DENV, including entomological surveys of confirmed cases, started in 2015, joining the efforts of different public organisations to carry it out. We present the surveillance conducted around viraemic travellers to early detect DENV in mosquitoes in order to avoid and control potential dengue autochthonous outbreaks.

**Methods**

**Epidemiological surveillance**

In Spain, DENV is monitored at the national level, in addition Catalonia has a regional epidemiological DENV surveillance. Following national and regional guidelines for arbovirus surveillance, travellers returning from endemic areas with a clinical syndrome compatible with an arboviral infection were screened for DENV and CHIKV in 2015. The arbovirus surveillance, which takes place during the period of *Ae. albopictus* activity, usually lasts from May to November, however, depending on the entomological situation in a given year, it can be expanded. In this study, travellers screened between April and December in Catalonia are included. The clinical and laboratory notification of cases was submitted to the local public health agency, which in turn alerted the mosquito control services.

The regional protocol for surveillance included patients with a febrile syndrome of less than 7 days of duration returning from tropical and subtropical areas. Samples were submitted for testing to the Microbiology Department at Hospital Clinic of Barcelona (centralised laboratory for testing) which reports to the local public health agency any positive RT-PCR or IgM test. During the first week after the onset of symptoms, serum

### Table

<table>
<thead>
<tr>
<th>Type of PCR and primers</th>
<th>Sequence</th>
<th>Localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td>EGENE2-S′</td>
<td>5′-CTGAAACATGGATGTCATCAGAAGG-3′</td>
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<tr>
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<td>RRT2</td>
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<tr>
<td></td>
<td>RN2</td>
<td>5′-CGKGARTTTCATYCCIATCGT-3′</td>
</tr>
</tbody>
</table>

* The genome positions are given according to each dengue virus serotype prototype Jamaica N-1409 strain (M20558).

This primer was described by Domingo et al. [43].
samples were tested for the presence of DENV RNA. In addition, after the day 4 post-symptom onset, serum samples were also tested for the presence of IgM and IgG against DENV.

Molecular diagnosis for DENV was performed using a specific real time-RT-PCR [20] that discriminates the DENV serotypes. Generic flavivirus RT-PCR [21] followed by sequencing and a dengue NS1-IgM-IgG detection rapid test (SD diagnostics Inc., Seoul, Korea) were eventually used when additional diagnostic evidence was needed. Antibodies against DENV were detected by a commercial ELISA kit (Panbio dengue IgM Capture ELISA and dengue IgG Indirect ELISA).

The following case definitions were applied in the regional protocol for arbovirus surveillance. A probable dengue case was a patient who had travelled to a dengue endemic area and presented with (i) fever and at least two of the following symptoms: anorexia, a positive Tourniquet test, arthralgia, leukopenia, myalgia, nausea, rash, any warning sign such as abdominal pain, persistent vomiting or mucosal bleeding and/or (ii) had a positive IgM against DENV. A confirmed dengue case was a probable case who has been laboratory confirmed through virus isolation, detection of the viral genome by RT-PCR or by seroconversion in a second sample collected 2–3 weeks after the first sample. For investigation of autochthonous cases, patients with similar compatible symptoms but with no history of travel to tropical and subtropical areas in the last 30 days were considered.

From September 2015, dengue became a mandatorily notifiable disease in Catalonia. The data used in the present study were retrieved from the records of the centralised laboratory in charge of DENV testing for the arbovirus regional surveillance programme.

**Entomological surveillance**

Entomological inspections were carried out by the Agència de Salut Pública de Barcelona (ASPB) in the city of Barcelona, by the Servei de Control de Mosquits del Consell Comarcal del Baix Llobregat (SCM) in the province of Barcelona, by Servei de Control de Mosquits Badia de Roses i Baix Ter in the Girona province and
by Consorci de Polítiques Ambientals de les Terres de l’Ebre (COPATE) in the Tarragona and Lleida provinces. The entomological inspections were performed in a radius of ca 100 m around the home of each diagnosed DENV viraemic case. This distance was selected based on the flight range of *Ae. albopictus* [22]. Inspection of the home address was conducted only with permission of the owner and within 10 days after DENV diagnostic, which was considered the duration of the viremia. When permission was not obtained, mosquitoes were sampled in the neighbourhood of the residence when possible. Other places such as workplace were inspected if these had been reported to be visited by the case during the viraemic period.

Entomological monitoring activities were carried out during the activity period of *Ae. albopictus* in the region, which usually starts in May and tapers off in November with activity peaks in July–September, in agreement with the *Ae. albopictus* phenology in other Mediterranean countries [8]. Adult mosquitoes were captured using BG Sentinel traps (Biogents GmbH, Regensburg, Germany) during time intervals of 24 or 48 hours, as well as entomological aspirators (Improved Prokopack Aspirator, Mod. 1419, John W. Hock Company, Florida, United States (US) and CDC Backpack Aspirator Mod. 2846, BioQuip, California (CA), US).

All accessible breeding larval sites were identified and treated if possible using a formulation of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* (Vectomax FG, Valent Biosciences Corporation, Libertyville, Illinois (Ill), US). Dissemination of information on mosquito prevention and control was conducted in the neighbourhood of viraemic cases’ residences, including instructions on how to eliminate mosquito breeding sites. When high densities of adults were found (continuous presence of biting females during the surveys), adulticide treatments were conducted if they were considered to be effective in terms of mosquito population reduction.

The female mosquitoes, captured between June and November 2015 in the context of this study, were identified and pooled into groups of up to 35 individuals according to the date and site of collection. The mosquito pools were kept in cell culture media (DMEM supplemented with 6% penicillin/streptomycin) and sent refrigerated to the laboratory at Centre de Recerca en Sanitat Animal (CReSA) for DENV detection.

**Figure 2**

Chronological representation of events leading to dengue virus detection in human and in *Aedes albopictus* samples, Catalonia, Spain, 2015

DENV: dengue virus.

Dengue virus detection from mosquitoes

All mosquitoes collected were quickly processed for the detection of DENV genome. The mosquito samples were homogenised and the viral RNA was extracted using NucleoSpin Virus kit according to the manufacturer’s instructions (NucleoSpin Virus kit, Macherey-Nagel, Düren, Germany). The mosquito pools were screened for DENV using a real-time TaqMan RT-PCR (RRT-qPCR) which allows detecting any DENV serotype using primers and a probe previously described [23]. Amplification was performed using the AgPath One-Step ID RTPCR kit (Ambion-Applied Biosystems, Foster City, CA, US) following the manufacturer’s instructions in Fast7500 equipment (Life Technologies, Austin, Texas (TX), US). Positive and negative controls for both extraction and amplification were used.
Figure 3
Investigation of the origin of a dengue virus serotype 2 detected in *Aedes albopictus* by phylogenetic analysis of the envelope gene sequence, Catalonia, Spain, September 2015

Strains are denoted by GenBank accession number, place and year of isolation. The green dots indicate the human and mosquito sequences obtained in our study, which were respectively submitted to GenBank under the accession numbers MH253297 and MH253296. The scale bar indicates substitutions per site.
The supernatant from the homogenised mosquito pool that resulted positive was sent to the National Reference Laboratory Centro Nacional de Microbiología (Instituto de Salud Carlos III, Madrid) for confirmation. The viral RNA was extracted using the QIamp Viral RNA Mini kit according to manufacturer instructions (Qiagen GmbH, Hilden, Germany). Two different RT-PCR assays were used for DENV detection: an in-house real-time RT-PCR and a conventional RT-nested-PCR [24].

In order to identify the DENV serotype, genetic material from the positive mosquito pool was partially sequenced using the cFD2 and MAMD primers previously described and specific for a fragment of the non-structural protein 5 (NS5) gene [25]. The amplification product was detected by electrophoresis and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing reactions were performed with ABI Prism BigDye terminator Cycle Sequencing v.3.1 Ready Reaction (Life Technologies, Austin, TX, US), and analysed using an ABI PRISM model 3730 automated sequencer (Life Technologies, Austin, TX, US). Comparisons with published sequences were performed by searches with the basic local alignment search tool (BLAST) programme at the National Center for Biotechnology Information (NCBI) against the complete GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) to identify the detected agent.

### Dengue virus characterisation and phylogenetic analysis

For genotyping and phylogenetic analysis, the envelope (E) gene of both mosquito and human samples was amplified using the primers described in Table 1. Amplification was performed using a one-step RT-PCR kit (Qiagen GmbH, Hilden, Germany) following the manufacturer’s instructions. A total of 5 µL of viral RNA was added to 45 µL RT-PCR mix. The RT-PCR mix contained 1x OneStep RT-PCR buffer, 400 mM of each dNTP, and 20 pmol of each primer. RT-PCR reactions were carried out using an initial reverse transcription step at 50 °C for 30 min followed by a denaturation (94 °C, 15 min) and 40 cycles including a denaturation (94 °C, 30 s), primer annealing (50 °C, 1 min), and primer extension (72 °C, 2.5 min) step. A final incubation was carried out at 72 °C for 10 min. A second amplification reaction (nested PCR) was seeded with 1 µL of the initial amplification product. Amplification was performed using Go Taq PCR kit (Promega, Madison, Wisconsin, US) following the manufacturer’s instructions and 20 pmol of each primer. Nested-PCR reactions were carried out using an initial denaturation (94 °C, 2 min) and 40 cycles of denaturation (94 °C, 30 s), primer annealing (50 °C, 45 s), and primer extension (72 °C, 2.5 min). A final incubation was carried out at 72 °C for 5 min. A phylogenetic tree was constructed with the common amplified E gene fragment (1,398 nt). The sequences were aligned by MUSCLE, and evolutionary distances were calculated by Tamura–Nei (TN93+I) model. Phylogenetic dendrograms were constructed using the maximum likelihood method and bootstrap analysis (1,000 replicates) by the Molecular Evolutionary Genetics Analysis programme (MEGA, Version 7).

The sequences of the E gene obtained in the study were submitted to GenBank Nt Sequence Database under the following accession numbers: MH253296 and MH253297.

### Results

During 2015, 131 patients were screened for DENV in Catalonia. Sixty-six patients resulted negative by RT-PCR and IgM testing and 65 dengue cases were detected between April and December. Of these, most cases (n = 23) were detected in July. Twenty-eight of these patients were viraemic, as demonstrated by the presence of DENV RNA in serum samples (dengue confirmed cases). In addition, IgM antibodies against DENV were detected in 37 patients, who were classified as probable dengue cases. Viraemic cases presented with a febrile syndrome and had visited at least one of the following countries: Colombia, Costa Rica, Dominican Republic, El Salvador, Honduras, Indonesia, Malaysia, Myanmar/Burma, Philippines, Sri Lanka, Thailand. For the cases with a positive IgM, the clinico-epidemiological data were incomplete.

Entomological inspections around the DENV viraemic cases were performed in a mean of 12 days after symptom onset (range: 1–18 days) that often started before the arrival to the country and subsequent visit to medical care. During these inspections, female mosquitoes could be trapped in relation to 17 of the 28 cases, either because mosquitoes were present or because access to the mosquito breeding sites was possible (Figure 1). Most breeding sites were abandoned objects with water, including toys, flowerpots or different kinds of scuppers. Six hundred females were obtained from 65 capture sessions performed by BG traps and entomological aspirations. The number of female *Ae. albopictus* mosquitoes per pool ranged from one to 35. The number of mosquito samples per localisation ranged from one to four.

Of all the mosquitoes collected, only one mosquito pool sampled in the Baix Llobregat region, Barcelona province, on 10 September 2015, was DENV positive (Figures 1 and 2). This pool was retrieved from the residence of a man in his 50s who had travelled to El Salvador. Upon returning from the trip he developed fever, malaise and arthralgia and sought medical attention. On 3 September 2015, serum samples from the patient were submitted to the clinical microbiology laboratory of the Hospital Clinic of Barcelona through the regional surveillance programme for arboviruses in Catalonia. As the samples were collected within 3 days of symptom onset, they were screened for DENV and CHIKV using specific real-time RT-PCR techniques. DENV serotype 2 was detected with a cycle threshold value (Ct) of 16 in serum. On 10 September, an entomological investigation was performed at the patient's
residence, a ground floor house where mosquitoes were abundant.

Two female mosquito pools were collected from the front patio, with 29 and three mosquitoes in each. The first one came from a BG Sentinel trap and the second one from aspiration. The mosquito pool with 29 Ae. albopictus females was DENV positive by RRT-qPCR with a Ct of 23.05. The homogenate of the DENV-positive mosquito sample was also confirmed to be positive by two different molecular assays at the laboratory of Arboviruses and Viral Imported Diseases in the Centro Nacional de Microbiología (Instituto de Salud Carlos III, Madrid).

The DENV detected in the mosquito sample was characterised as DENV serotype 2, as a fragment of its NS5 gene sequence showed a high similarity to those of several DENV serotype 2 isolates from South America. Phylogenetic analysis based on E gene sequences confirmed the identity of the DENV sequences detected in both mosquitoes and patient. The DENV-2 detected was related to other strains of the American/Asia genotype circulating in the Americas (Figure 3).

Because a person who lived in the same house as the viraemic patient was diagnosed with multiple chemical hypersensitivity syndrome, no chemical treatments were conducted in the house’s patio, although they were carried out in similar patios from contiguous houses. Adults were removed using traps and by aspirations. All possible breeding sites were eliminated. Larvicidal treatments using a formulation of Bacillus thuringiensis israelensis and Bacillus sphaericus (Vectormax FG, Valent Biosciences Corporation, Libertyville, Ill, US) in all scuppers on the streets in a radius of 100 m around the house of the patient, and adulticide treatments using deltamethrin were performed in contiguous houses’ patio and in an abandoned plot situated in the back of the houses. On the days following the finding of DENV-positive mosquito pools (16, 18 and 21 September) a great effort was made to further sample mosquitoes in the patient’s home and more Ae. albopictus (237 females) were collected using BG traps and aspirations, from the same location, all them resulting negative for DENV. Moreover, no autochthonous transmission in humans was reported in this location during the rest of the mosquito activity season in 2015.

Discussion

The present study shows how epidemiological arbovirus surveillance, including mosquito collection around dengue viraemic cases, can be useful to early detect DENV circulation in local Ae. albopictus mosquitoes. The finding of a high viral load of DENV in mosquitoes present at the Baix Llobregat region evidenced that the level of viraemia in the infected traveller from El Salvador was sufficient to efficiently infect local Ae. albopictus, highlighting the risk of DENV autochthonous transmission in this area.

Ae. albopictus population density, vector competence, blood feeding behaviour and vector longevity would be determinants for both human-to-mosquito and mosquito-to-human transmission of dengue in Europe. The risk of dengue importation into Europe is greatest in August to October due to a high mosquito activity in that period and a number of passengers coming from dengue-affected areas, some of whom possibly in the viraemic phase [26]. Semenza et al. [26] pointed out that Barcelona, where Ae. albopictus is present, is one of the three large European cities with higher risk of dengue transmission. The present study, which finds DENV in Ae. albopictus from Barcelona province provides further evidence to this.

Different DENV strains have been involved in autochthonous transmission in Europe since 2010, and all belonged to either serotype 1 or serotype 2 [16,17,27-29]. DENV serotypes 1 and 2 have been the most prevalent DENV serotypes in travellers to Europe during the last years [30]. In the present study, the DENV serotype 2 was characterised as originating from America in both mosquito and human sera samples.

Ae. albopictus can only be unequivocally incriminated as a vector of dengue where transmission occurs in the absence of Ae. aegypti or any other potential vector [31]. However, to our knowledge, although Ae. albopictus has been implicated as a vector of DENV in all the recent mainland European autochthonous transmissions no DENV detection in local mosquitoes has been reported before our finding. Despite seemingly favourable conditions, places where Ae. albopictus predominates over Ae. aegypti have never experienced a typical explosive dengue epidemic with severe cases of the disease [32]. Although Ae. albopictus is overall more susceptible to DENV mid gut infection, rates of virus dissemination from the mid-gut to other tissues are significantly lower in Ae. aegypti than in Ae. albopictus. Therefore, Ae. albopictus may play a relatively minor role compared with Ae. aegypti in DENV transmission, and this could also be partly due to differences in feeding preferences (i.e. feeding on humans and animals vs humans only) and reduced vector competence [32]. This statement would explain the European autochthonous DENV transmissions in Croatia [27] and France [13,17], as well as those recently detected in Spain in 2018 [33], where Ae. albopictus was the suspected vector. Moreover this explanation would also concur with the findings of our study, whereby DENV was detected in Ae. albopictus mosquitoes around the imported case in Catalonia, but no autochthonous cases occurred.

Nevertheless, recent examples of rapid arboviral adaptation to alternative mosquito vectors as occurred in the chikungunya outbreak in Indian Ocean islands in 2005 [34] should be taken into account and the possibility of a DENV evolution to be efficiently transmitted by Ae. albopictus not disconsidered. Moreover, as already reported for DENV, different vector competence
of different mosquito populations from the same mosquito species can exist [35,36]. Therefore, vector competence studies for DENV in different European Ae. albopictus mosquito populations such as those reported by Vega-Rúa et al. [37], in a mosquito population of France, which showed high efficiency to transmit DENV, will be useful to better estimate the risk of a DENV autochthonous outbreak in a certain area. In fact, a recent study has experimentally shown that an Ae. albopictus strain from Catalonia could sustain DENV-2 replication under a simulated Mediterranean temperature regime [38], which would be in agreement with our detection of a high viral load in the DENV-positive mosquito pool in the summer 2015.

Integrated vector management techniques such as source reduction, public education, pesticide application and biological control, produce the optimal control strategy [39], but with limited success if there is a poor participation of communities and a lack of coordination and synchronised implementation [40]. An integrated Aedes mosquito control strategy requires the coordinated involvement of local authorities, private partners, organised society and communities. In addition to surveillance of Ae. albopictus, maintaining vigilance for any introduction of Ae. aegypti species is essential, to assess the risk of mosquito borne diseases and to prepare for the control of disease outbreaks [9].

Early arbovirus detection and effective public health measures reduce the risk of a wider mosquito-borne diseases distribution and increased health impact on the public. Moreover, given the unpredictability of vector-borne disease outbreaks in terms of time, these measures will offer relevant, target-oriented strategies to mitigate problems from their beginning [41]. In case of a dramatic increase of imported viraemic cases, an integrated surveillance could be sustainable if a well-established entomological surveillance framework in coordination with public health authorities was maintained in time. The results of the present study point out that an early intervention is able to detect mosquito infection and to likely reduce the risk of autochthonous transmission as previously recommended by Rezza [42] for areas with relatively cold and dry winters.

The present study highlights the success of an early DENV detection in both human and mosquitoes and the effectiveness of an epidemiological surveillance, which combines epidemiology with ecology, in interrupting local DENV transmission as described by Hubaleck [3]. To sum up, this arbovirus surveillance consisted of different components: (i) routine diagnosis of human disease, (ii) epidemiological investigation, (iii) monitoring and control of Ae. albopictus population and (iv) testing mosquito vectors for DENV. However, this surveillance had some limitations: First, complete clinical and epidemiological data were not available for some probable cases and therefore we could not confirm whether they fulfilled all criteria to be classified as dengue cases. On the other hand, the travel history was available for confirmed cases and the data on the viraemic patient and associated DENV-positive mosquitoes were clear. Second, it is possible that asymptomatic dengue cases (imported or autochthonous) may remain undetected. Third, in some instances performing entomological inspections to test mosquitoes for DENV can be difficult due to limited access of the of viraemic patients’ residences.

Arbovirus surveillance relies on multidisciplinarity activities, which are essential for an early detection and prompt implementation of control measures to avoid local arbovirus transmission. Moreover, community engagement is necessary to early detect Ae. albopictus in areas where it is not yet established and control its population. Therefore, public health professionals responsible for managing disease outbreaks and policymakers working together with experts from different fields such as virologists, molecular biologists, epidemiologists and entomologists and society as a whole could guarantee a better mosquito-borne disease surveillance. On the other hand, epidemiological surveillances require fluid communication within all the surveillance components and substantial resources that are not always available.

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Conflict of interest

None declared.

Authors’ contributions


Conflict of interest

None declared.

Authors’ contributions

Conceived and designed the study: CA, MM, TM, IC, EB, LP, MI, IB, NT, and NB; Sample collection: CA, TM, REritja, EH, EM and REScosa. Data processing and analysis: CA, MM, TM, MJ, ST, AV, MS and NB. Drafting the manuscript: CA, MM, TM, REritja, NT and NB. All authors gave final approval of the version to be submitted.

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A comparison of two biological markers of recent hepatitis C virus (HCV) infection: implications for the monitoring of interventions and strategies to reduce HCV transmission among people who inject drugs

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Background: Monitoring hepatitis C virus (HCV) incidence is important for assessing intervention impact. Longitudinal studies of people who inject drugs (PWID), using repeated biological tests, are costly; alternatively, incidence can be estimated using biological markers of recent infection in cross-sectional studies. Aim: We aimed to compare incidence estimates obtained from two different biological markers of recent infection in a cross-sectional study to inform monitoring approaches for HCV elimination strategies. Method: Samples from an unlinked anonymous bio-behavioural survey of PWID were tested for two recent infection markers: HCV RNA with anti-HCV negative (‘RNA’) and low-avidity anti-HCV with HCV RNA present (‘avidity’). These two markers were used separately and in combination to estimate HCV incidence. Results: Between 2011 and 2013, 2,816 anti-HIV-negative PWID (25% female) who had injected during the preceding year were either HCV-negative or had one of the two markers of recent infection: 57 (2.0%) had the RNA marker and 90 (3.2%) the avidity marker. The two markers had similar distributions of risk and demographic factors. Pooled estimated incidence was 12.3 per 100 person-years (pyrs) (95% credible interval: 8.8–17.0) and not significantly different to avidity-only (p = 0.865) and RNA-only (p = 0.691) estimates. However, the RNA marker is limited by its short duration before anti-HCV seroconversion and the avidity marker by uncertainty around its duration. Conclusion: Both markers have utility in monitoring HCV incidence among PWID. When HCV transmission is high, one marker may provide an accurate estimate of incidence; when it is low or decreasing, a combination may be required.

Introduction
In high-income countries, hepatitis C virus (HCV) transmission is focused among people who inject drugs (PWID), with infection often acquired soon after initiation to injecting [1,2]. In the United Kingdom (UK), it is estimated that around half of PWID have been infected with HCV [3], although there is considerable geographical variation in prevalence [4]. Estimates of HCV incidence among PWID are high, typically between 10 and 20 infections per 100 person-years (pyrs) of exposure [5].

Measuring incidence, and how this changes over time, is important for assessing the impact of interventions to prevent and control HCV, such as needle and syringe programmes (NSPs), opioid substitution therapy (OST) and HCV treatment as prevention (HCV TasP), as well as monitoring progress towards the global goal of eliminating HCV as a major public health threat [6]. Incidence is traditionally estimated through longitudinal follow-up studies; however, such studies are expensive and difficult among PWID because follow-up is hindered by the illicit nature of drug use and the marginalisation of PWID [7]. An alternative approach is to use biological markers of recent infection in cross-sectional studies. Two approaches have been advocated.

The first approach involves the detection of HCV RNA among individuals found to be HCV antibody (anti-HCV)-negative, indicating an acute and therefore
In order to better understand the determinants underlying the variation in HCV incidence among PWID, we undertook an analysis to compare the estimated incidence, and associated risk factors, for the two different approaches of estimating incidence using biological markers (HCV RNA in antibody-negative individuals and antibody avidity in antibody-positive individuals with HCV RNA) using pooled data from a large national bio-behavioural survey. We also estimate overall incidence based on the two approaches accounting for the uncertainty in their window periods. The findings are important for informing the choice of optimal method for monitoring HCV incidence among PWID in the UK and elsewhere.

Methods

Survey
PWID across England, Wales and Northern Ireland are recruited into an annual cross-sectional, unlinked anonymous bio-behavioural survey (the UAM Survey); methodological details have been previously reported [23,24]. In brief, people who have ever injected drugs are recruited through specialist services for PWID providing advice, NSPs, OST or addiction treatment. Service selection reflects the range of services provided for PWID and what is known about geographic variations in drug use. Those agreeing to participate self-complete a short questionnaire and provide a dried-blood spot (DBS) sample at the collaborating service. DBS collection involves obtaining a few drops of blood, through a lancet prick to the finger, onto absorbent filter paper (PerkinElmer 226). The survey has multi-site ethical approval.

In addition to core demographics (age and gender), the questionnaire collects self-reported behavioural data, including prior imprisonment and homelessness, types of psychoactive drug used, injecting risks, uptake of health services (e.g. OST), and sexual behaviours (e.g. condom use). In this study, we included only individuals recruited between 2011 and 2013 inclusive who had injected during the year preceding survey participation.

Testing for recent infection markers
The DBS samples were tested for antibodies to HIV (anti-HIV), hepatitis C (anti-HCV) and hepatitis B core antibody (anti-HBc). All laboratory testing was carried out at the Virus Reference Department at Public Health England, Colindale, using previously reported methods [8,13]. Two methods were applied to identify recent infections: anti-HCV avidity testing algorithm (upon receipt at laboratory) and RNA testing of the anti-HCV negative samples (on stored samples, with testing undertaken during 2014–15). A 6 mm spot was used for serological and molecular testing.

Anti-HCV avidity testing
The method was undertaken as previously described [13,18]. Briefly, each sample is tested in duplicate
with one well incubated with urea (avidity well) and the second with wash buffer (control well). In the presence of urea, low-avidity (weakly bound) antibodies will dissociate from HCV antigen bound to the solid phase. An avidity index (AI) is determined for each specimen (optical density (OD) urea-treated/OD untreated) × 100, with an AI ≤ 40% considered to be low. As low avidity antibody can also be found in individuals who have cleared HCV RNA, specimens with low AI were subsequently tested for HCV RNA using PCR [13]. Those individuals with DBS containing both low-avidity anti-HCV and HCV RNA were considered to have recently acquired their HCV infection (i.e. to have markers compatible with recent primary infection).

HCV RNA testing of those anti-HCV-negative

To identify those participants whose samples were anti-HCV-negative and HCV RNA-positive, stored residual DBS samples from those found to be anti-HCV-negative when tested on receipt underwent retrospective HCV RNA testing.

Table 1
Markers of recent hepatitis C infection\(^a\) according to survey year, demographics and risk factor variables, among people who inject drugs, England, Wales and Northern Ireland, 2011–13 (n = 2,816)

<table>
<thead>
<tr>
<th>Variable</th>
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<th>Avidity marker of recent infection</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Positive %</td>
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<tr>
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<td></td>
<td>2012</td>
<td>969</td>
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<td>2013</td>
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<tr>
<td>Age</td>
<td>&lt;30</td>
<td>957</td>
<td>56</td>
<td>5.9</td>
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</tr>
<tr>
<td></td>
<td>30–39</td>
<td>1,277</td>
<td>62</td>
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<tr>
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<td>≥40</td>
<td>582</td>
<td>29</td>
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<td>2,099</td>
<td>101</td>
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<tr>
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<td>Female</td>
<td>717</td>
<td>46</td>
<td>6.4</td>
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<tr>
<td>Regional prevalence</td>
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<td>1,114</td>
<td>50</td>
<td>4.5</td>
<td>26</td>
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<tr>
<td></td>
<td>Medium (40–55%)</td>
<td>768</td>
<td>35</td>
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<td>High (&gt;55%)</td>
<td>934</td>
<td>62</td>
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<tr>
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<tr>
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<td>1–5 years</td>
<td>783</td>
<td>39</td>
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<td>6–10 years</td>
<td>591</td>
<td>36</td>
<td>6.1</td>
<td>12</td>
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<tr>
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<td>11–14 years</td>
<td>499</td>
<td>22</td>
<td>4.4</td>
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<td>15–19 years</td>
<td>457</td>
<td>20</td>
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<td>7</td>
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<tr>
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<td>≥20 years</td>
<td>329</td>
<td>21</td>
<td>6.4</td>
<td>4</td>
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<tr>
<td>Homelessness</td>
<td>Never</td>
<td>696</td>
<td>23</td>
<td>3.3</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Yes, not past year</td>
<td>1,063</td>
<td>58</td>
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<tr>
<td></td>
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<td>65</td>
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<td>22</td>
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<td></td>
<td>1–4 times</td>
<td>886</td>
<td>33</td>
<td>3.7</td>
<td>14</td>
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<tr>
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<td>≥5 times</td>
<td>801</td>
<td>60</td>
<td>7.5</td>
<td>21</td>
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<tr>
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<td>3.8</td>
<td>37</td>
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<tr>
<td></td>
<td>Yes</td>
<td>548</td>
<td>60</td>
<td>10.9</td>
<td>20</td>
</tr>
<tr>
<td>Speed (amphetamine) injecting</td>
<td>No</td>
<td>2,335</td>
<td>127</td>
<td>5.4</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>481</td>
<td>20</td>
<td>4.2</td>
<td>8</td>
</tr>
<tr>
<td>NSP use</td>
<td>Never/not in past year</td>
<td>499</td>
<td>25</td>
<td>5.0</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>In past year</td>
<td>2,300</td>
<td>121</td>
<td>5.3</td>
<td>48</td>
</tr>
<tr>
<td>Injecting and sharing past month</td>
<td>Did not inject past month</td>
<td>674</td>
<td>18</td>
<td>2.7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Injected and did not share</td>
<td>1,418</td>
<td>59</td>
<td>4.2</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Injected and shared</td>
<td>724</td>
<td>70</td>
<td>9.7</td>
<td>26</td>
</tr>
</tbody>
</table>

NSP: needle and syringe programmes.
\(^a\) Antibody-negative with RNA and weak avidity of antibody.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Logistic model</th>
<th>Multinomial logistic model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Combined OR (95% CI)</td>
<td>RNA RRR (95% CI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p value</td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>1.46 (0.94–2.27)</td>
<td>1.00 (0.50–1.99)</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>1.29 (0.83–2.00)</td>
<td>1.21 (0.63–2.33)</td>
</tr>
<tr>
<td>Age</td>
<td>30</td>
<td>1.22 (0.84–1.77)</td>
<td>2.06 (1.15–3.69)</td>
</tr>
<tr>
<td></td>
<td>30–39</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
</tr>
<tr>
<td></td>
<td>≥40</td>
<td>1.03 (0.65–1.62)</td>
<td>1.04 (0.47–2.31)</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>1.36 (0.95–1.94)</td>
<td>1.49 (0.85–2.60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regional prevalence</td>
<td>Low (40%)</td>
<td>0.98 (0.63–1.53)</td>
<td>1.63 (0.80–3.32)</td>
</tr>
<tr>
<td></td>
<td>Medium (40–55%)</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
</tr>
<tr>
<td></td>
<td>High (55%)</td>
<td>1.49 (0.97–2.28)</td>
<td>1.53 (0.73–3.21)</td>
</tr>
<tr>
<td>Injecting duration</td>
<td>&lt;1 year</td>
<td>0.94 (0.44–1.99)</td>
<td>0.94 (0.26–3.37)</td>
</tr>
<tr>
<td></td>
<td>1–5 years</td>
<td>0.81 (0.51–1.29)</td>
<td>1.49 (0.74–3.01)</td>
</tr>
<tr>
<td></td>
<td>6–10 years</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
</tr>
<tr>
<td></td>
<td>11–14 years</td>
<td>0.71 (0.41–1.23)</td>
<td>0.68 (0.27–1.74)</td>
</tr>
<tr>
<td></td>
<td>15–19 years</td>
<td>0.71 (0.40–1.24)</td>
<td>0.74 (0.29–1.90)</td>
</tr>
<tr>
<td></td>
<td>≥20 years</td>
<td>1.05 (0.60–1.83)</td>
<td>0.60 (0.19–1.88)</td>
</tr>
<tr>
<td>Homelessness</td>
<td>Never</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
</tr>
<tr>
<td></td>
<td>Yes, not past year</td>
<td>1.69 (1.03–2.76)</td>
<td>1.46 (0.71–3.00)</td>
</tr>
<tr>
<td></td>
<td>Yes, past year</td>
<td>2.02 (1.24–3.28)</td>
<td>1.43 (0.69–2.97)</td>
</tr>
</tbody>
</table>

CI: Confidence interval; OR: odds ratio; ref: reference value; RRR: relative risk ratio.

<sup>a</sup> RNA-positive antibody-negative (RNA marker) and antibody-positive with weak avidity (avidity marker).

<sup>b</sup> p value diff is the p value for the effect estimates for RNA and avidity being the same.
RNA testing involved elution from the DBS by incubating the 6 mm spot for 2 hours at 56 °C with 20 µL of proteinase K and 300 µL of ATL lysis buffer (Qiagen products: 19133 and 19076). The entire eluate was extracted on the Qiagen Biorobot MDX platform using the QIAamp One-For-All Nucleic Acid Kit (Qiagen product: 965672) and One For All MDx cV70a protocol. Brome mosaic virus (BMV) was added as the internal control. Amplification and detection of HCV RNA and BMV was undertaken as previously described [25], employing a PCR targeting the non-coding region of the HCV genome.

These two approaches identified those recently infected at two different stages, which are mutually exclusive, in the sequence from being uninfected to having established infection (Figure 1).

Samples that were anti-HIV positive (n = 25) were excluded, as the effects of HIV on the immune system is likely to affect anti-HCV avidity [20]. Recent HCV infection status was based on the two markers, (i) RNA-positive antibody-negative (henceforth ‘RNA’), and (ii) HCV antibodies with weak avidity in those with HCV RNA (henceforth ‘avidity’). The analytical dataset consisted of individuals who had one of these two markers of recent infection or who were susceptible to HCV infection (uninfected, defined as anti-HCV- and RNA-negative); i.e. those with established infection were excluded. Analysing such data via logistic regression-type models will therefore provide odds ratios (OR) for recent infection according to the two markers.

### Statistical analysis

Patterns of recent infection were examined according to a number of demographic, geographic and risk factor covariates. The demographic variables were year of test (2012 and 2013 vs 2011), age (<30 and ≥40 vs 30–39) and gender (female vs male). The geographic variable was regional prevalence groups that were
*Antibody-negative with RNA (RNA marker) and weak-avidity antibody (avidity marker).*

95% confidence intervals are indicated by solid and dashed lines.

Based on overall HCV antibody prevalence from 2011 to 2013, with low defined as ≤40% (South West, North East, Wales and Northern Ireland); medium as 40–55% (East of England, West Midlands, East Midlands) and high as >55% (London, North West, South East, Yorkshire and the Humber). The risk factor variables were years since first injection (1, 1–5, 11–14, 15–19 and ≥20 vs 6–10), homelessness (‘yes, not in past year’ vs never), imprisonment (1–4 times and ≥5 times vs never), injecting crack in the past month (yes vs no/did not inject in past month/unknown), injecting speed in the past month (yes vs no/did not inject in past month/unknown) and injecting and equipment sharing behaviour in the past month (derived from two sequential questions). The latter group was defined as did not inject in past month, injected in past month but did not share equipment, and injected and shared equipment in past month, and therefore incorporates the difference in risk between those that injected in the past month and those that did not.

A combined outcome for recent infection according to either the RNA or avidity measure was examined in relation to the factors above via logistic regression to estimate ORs for any marker of recent infection vs susceptible. In addition, the RNA and avidity measures of recent infection were each analysed as distinct outcomes (vs those susceptible) in a multinomial logistic model; this allowed the effect of the covariate for the separate outcomes to be modelled in terms of relative risk ratios (RRR). The multinomial model allowed for testing the equivalence of parameters in the model for the two recent infection markers, i.e. whether the two markers provided consistent estimates of the risk factors that are predictive of recent infection. In this context, RRRs and ORs are comparable; in particular, if RRRs for the two markers are identical they will be exactly equal to the OR for the combined outcome.

Univariable analyses were conducted and a multivariable model constructed on the basis of backwards stepwise variable selection with a p value of 0.2 for removal. Variables were selected for the logistic and multinomial logistic models and any variables retained in either model were included in the final set of variables. Analyses were conducted using Stata version 13.1 (StataCorp, College Station, Texas, United States). We estimated incidence based on markers of recent infection using the formula: \( I = \frac{r}{wn} \),

where \( I \) is the incidence rate, \( w \) the length of the window period, \( r \) the number of individuals with the marker of recent infection and \( n \) the number not infected [26]. The uncertainty arising from both sampling variability of the binomial data (\( r, n \)) and the length of the window period were accounted for using a fully Bayesian approach. We specified uniform priors for the window period, ranging from 51 to 75 days for RNA and from 60 to 180 days for avidity. For the latter, we also examined a semi-informative beta(2,2) distribution across the range of the 60–180 day window period, such that the interquartile range of the prior distribution was 99–141 days compared with 90–150 under a uniform prior. Medians of the posterior distributions were taken as point estimates and the 2.5th and 97.5th percentiles as 95% credible intervals (CrI). The model was implemented in WinBUGS version 1.4.3 (Medical Research Council, UK).

**Results**

Between 2011 and 2013, there were 2,816 anti-HIV negative participants who had injected during the preceding year whose samples were either anti-HCV-negative or had one of the two markers of a probable recent HCV infection. Of these, 57 (2.0%) were HCV RNA-positive and anti-HCV-negative (‘RNA’) and a further 90 (3.2%)
had weak anti-HCV avidity in the presence of HCV RNA ('avidity'). Overall, the mean age of the participants was 34 years (median: 34; interquartile range: 28–39) and 717 (25%) were female. Table 1 shows numbers of individuals with each marker of infection according to survey year, demographics and risk factor variables.

Associations between risk factors and markers of recent infection

The univariable results from the model (Table 2) are summarised in Figure 2. Year of test and gender were not associated with either measure of incidence. There was evidence of an association (p = 0.03) with age for the RNA marker, with risk highest in those younger than 30 years, but there was no association for the avidity marker (p = 0.042 for inconsistency). There was some evidence of increased risk of recent infection for both markers in high-prevalence areas (OR = 1.49; 95% confidence interval (CI): 0.97–2.28; two markers combined).

Patterns of recent infection were inconsistent according to the two markers for injecting duration (p = 0.032 for inconsistency). For the RNA marker, there was a slightly higher rate in those injecting for 1 to 5 years vs 6 to 10 years (RRR = 1.19; 95% CI: 0.89–1.59), longer durations had non-significant lower rates. For the avidity marker, there were significantly lower rates for 1 to 5 years injecting vs 6 to 10 years (RRR = 0.90; 95% CI: 0.72–1.14) and a non-significant small increase for injecting for more than 10 years. For both markers, there was no evidence of a difference in risk for those injecting for less than 1 year.

Homelessness, being imprisoned five or more times and injecting crack all showed a higher risk of recent HCV infection, with consistent estimates for the two markers. Injecting in the past month showed a modest increase in risk, with a significant association for the avidity marker but not the RNA marker, although overall differences for the two markers again had non-significant p values for inconsistency. Sharing injecting equipment in the past month showed a large increase in risk for both markers of recent HCV infection.

The stepwise selection procedure included gender, region, injecting duration, homelessness, imprisonment, crack injecting and injected/shared injecting equipment in the past month in the final model (Figure 3 and Table 3). The ORs and RRRs were generally similar to the univariable results (Table 2), but risk factors with stronger associations were attenuated somewhat, such as homelessness (adjusted odds ratio (AOR) = 1.56 vs OR = 2.02 for homeless in last year, two markers combined) and sharing injecting equipment in the past month (AOR = 2.69 vs OR = 3.90, two markers combined). Interestingly, gender showed some evidence of an association in the adjusted model (AOR = 1.59; 95% CI: 1.07–2.37, two markers combined) but not in the univariable models.

Estimated incidence

The pooled estimate of incidence from the Bayesian model was 12.3 per 100 pyrs (95% CrI: 8.8–17.0) when using both markers of recent infection. As expected, the posterior distribution for the window period of RNA was near identical to the prior, i.e. uniformly distributed between 51 and 75 days. However, the posterior distribution for the weak-avidity window was shifted somewhat from the prior distribution to be consistent with the RNA marker data, with a median duration of 100 days (vs a mid-point of 120 days) and a 95% CrI of 68 to 148 days. Using RNA alone gave an incidence estimate of 12.7 per 100 pyrs (95% CrI: 9.1–17.7), very similar to the pooled estimate (p value for difference = 0.891). Using the avidity marker alone gave an incidence estimate of 11.8 per 100 pyrs (95% CrI: 6.6–21.3); this was imprecise owing to the uncertainty about the duration of the low-avidity period. With a semi-informative
Table 3
Multivariable model results from multinomial logistic model of markers of recent infection and logistic regression model of combined outcome, among people who inject drugs, England, Wales and Northern Ireland, 2011–13 (n = 2,816)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Combined OR (95% CI)</th>
<th>p value</th>
<th>RNA RRR (95% CI)</th>
<th>p value</th>
<th>Avidity RRR (95% CI)</th>
<th>p value</th>
<th>p value diff b</th>
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</thead>
<tbody>
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<td>Gender</td>
<td>Male</td>
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<td></td>
<td>1 (ref)</td>
<td></td>
<td>1 (ref)</td>
<td></td>
<td>0.072</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>1.59 (1.07–2.37)</td>
<td>0.022</td>
<td>1.55 (0.85–2.84)</td>
<td>0.152</td>
<td>1.61 (0.97–2.68)</td>
<td>0.072</td>
<td>0.962</td>
</tr>
<tr>
<td>Regional prevalence</td>
<td>Low (&lt;40%)</td>
<td>1.08 (0.69–1.71)</td>
<td>0.237</td>
<td>1.73 (0.83–3.57)</td>
<td>0.339</td>
<td>0.77 (0.42–1.38)</td>
<td>0.086</td>
<td>0.104</td>
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<td>1 (ref)</td>
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<td>1.40</td>
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</tr>
<tr>
<td></td>
<td>High (&gt;55%)</td>
<td>1.41 (0.91–2.18)</td>
<td></td>
<td>1.45 (0.68–3.06)</td>
<td>0.86</td>
<td>0.93 (0.57–1.52)</td>
<td>0.86</td>
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</tr>
<tr>
<td>Injecting duration c</td>
<td>&lt;1 year</td>
<td>1.00 (0.46–2.16)</td>
<td>0.790</td>
<td>1.01 (0.28–3.68)</td>
<td>0.099</td>
<td>0.99 (0.38–2.53)</td>
<td>0.99</td>
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</tr>
<tr>
<td></td>
<td>1–5 years</td>
<td>0.85 (0.52–1.38)</td>
<td></td>
<td>1.53 (0.75–3.12)</td>
<td>0.49</td>
<td>0.49 (0.25–0.96)</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6–10 years</td>
<td>1 (ref)</td>
<td></td>
<td>1 (ref)</td>
<td></td>
<td>1 (ref)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11–14 years</td>
<td>0.74 (0.42–1.29)</td>
<td></td>
<td>0.68 (0.26–1.75)</td>
<td>0.309</td>
<td>0.77 (0.39–1.51)</td>
<td>0.129</td>
<td>0.032</td>
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<tr>
<td></td>
<td>15–19 years</td>
<td>0.77 (0.43–1.38)</td>
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<td>0.78 (0.30–2.02)</td>
<td>0.77</td>
<td>0.77 (0.38–1.57)</td>
<td>0.77</td>
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<tr>
<td></td>
<td>≥20 years</td>
<td>1.09 (0.61–1.93)</td>
<td></td>
<td>0.61 (0.39–1.92)</td>
<td>0.134</td>
<td>1.34 (0.69–2.61)</td>
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</tr>
<tr>
<td>Homelessness</td>
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<td>1 (ref)</td>
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<td>1 (ref)</td>
<td></td>
<td>1 (ref)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes, not past year</td>
<td>1.49 (0.89–2.48)</td>
<td>0.237</td>
<td>1.38 (0.66–2.91)</td>
<td>0.631</td>
<td>1.61 (0.81–3.20)</td>
<td>0.103</td>
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<tr>
<td></td>
<td>Yes, past year</td>
<td>1.52 (0.92–2.51)</td>
<td></td>
<td>1.11 (0.52–2.36)</td>
<td>1.90</td>
<td>1.90 (0.97–3.71)</td>
<td>1.90</td>
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</tr>
<tr>
<td>Imprisonment</td>
<td>Never</td>
<td>1 (ref)</td>
<td></td>
<td>1 (ref)</td>
<td></td>
<td>1 (ref)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1–4 times</td>
<td>0.76 (0.48–1.20)</td>
<td>0.003</td>
<td>0.80 (0.40–1.61)</td>
<td>0.122</td>
<td>0.74 (0.41–1.35)</td>
<td>0.019</td>
<td>0.974</td>
</tr>
<tr>
<td></td>
<td>≥5 times</td>
<td>1.62 (1.06–2.48)</td>
<td></td>
<td>1.63 (0.83–3.17)</td>
<td>1.65</td>
<td>1.65 (0.96–2.81)</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>Crack injecting</td>
<td>No</td>
<td>1 (ref)</td>
<td></td>
<td>1 (ref)</td>
<td></td>
<td>1 (ref)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>2.32 (1.58–3.41)</td>
<td>&lt; 0.001</td>
<td>2.28 (1.22–4.24)</td>
<td>0.009</td>
<td>2.32 (1.45–3.72)</td>
<td>2.32</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Injecting and sharing past month</td>
<td>Did not inject past month</td>
<td>1 (ref)</td>
<td></td>
<td>1 (ref)</td>
<td></td>
<td>1 (ref)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Injected and did not share</td>
<td>1.20 (0.68–2.10)</td>
<td>&lt; 0.001</td>
<td>0.86 (0.39–1.89)</td>
<td>0.036</td>
<td>1.61 (0.73–3.59)</td>
<td>0.001</td>
<td>0.438</td>
</tr>
<tr>
<td></td>
<td>Injected and shared</td>
<td>2.69 (1.52–4.73)</td>
<td></td>
<td>1.83 (0.83–4.06)</td>
<td>3.79</td>
<td>3.79 (1.69–8.50)</td>
<td>3.79</td>
<td></td>
</tr>
</tbody>
</table>

CI: 95% confidence interval; OR: odds ratio; ref: reference value; RRR: relative risk ratio.

a RNA-positive antibody-negative (RNA marker) and antibody-positive with weak avidity (avidity marker).
b p value diff is the p value for the effect estimates for RNA and avidity being the same.

Using three duration categories 0–5, 6–14 and ≥15 years, with 6–14 years as baseline, the pattern is similar. For the 0–5 years category for RNA, RRR = 1.64 (95% CI: 0.91–2.98) and for avidity, RRR = 0.80 (95% CI: 0.38–1.70). For the ≥15 years category for the RNA, RRR = 0.62 (95% CI: 0.36–1.07) and for avidity, RRR = 1.07 (95% CI: 0.66–1.73).
beta distribution for the window period, the estimate was 11.1 per 100 pyrs (95% CrI: 7.0–18.9), a modest improvement in precision; this made no difference in the combined model. The avidity-only estimate (with informative window) was not significantly different to the pooled estimate (p = 0.691) or from the RNA-only estimate (p = 0.691).

Discussion
Our study is unique in comparing two biological markers of recent HCV infection in a large sample of community-recruited PWID. The factors associated with the recent infections identified by combining both markers, HCV RNA in those anti-HCV negative and HCV antibody avidity, were similar to those identified by each measure separately, and were generally expected associations with known HCV risk factors. In our combined model using both markers, the avidity marker contributed little information on absolute incidence rates owing to substantial uncertainty over the window period for this measure, with the combined result driven by the RNA data and its more certain window period. Nevertheless, incidence estimates from the two measures were very close, and the pooled analysis may give a better idea of the true window period for weak avidity, which we estimated at around 100 days on average, although 95% CrI were still wide, ranging from 68 to 148 days.

The factors considered here in relation to recent infection were a mixture of indicators of elevated risk, such as frequency of imprisonment [13], and risk behaviours representing a recent infection risk, such as sharing injecting equipment [21,27,28]. We found consistent patterns for the two markers (and when combined), with factors that have previously been shown to be associated with increased risk of HCV infection: female gender [28-31], imprisonment [13,32], the injection of crack [22,28] and the sharing of injecting equipment [33,34]. Results for injecting duration showed some inconsistency but in general, factors that showed a strong association with recent infection had consistent results for both markers.

Previous studies of risk factors for HCV and modelling work, including force of infection (FOI) estimates, have generally indicated an increased infection risk during the first year of injecting [28,35], in those who started injecting recently [27,28] or who had been injecting for only a few years [36]. We did not find such an association; however, our data are somewhat sparse, with just nine recent infections (i.e. having the RNA or avidity marker) in those injecting for less than one year; nevertheless, there should be sufficient power to detect a threefold or higher increase in risk. It is possible that some respondents imprecisely recalled their age at first injection, which would have led to an incorrect time since first injection and thereby some misclassification of recent infections. Alternatively, high excess risk at initiation may largely be due to the first few injecting events, when the individual may not have learned to inject themselves or started to use services [37], leading to these being under-represented in our service based sample. While our survey approach is established, the illicit and marginalised nature of injecting drug use makes construction of a formal sampling frame impossible.

The UK’s mature epidemics of injecting drug use and HCV have resulted in an ageing population of PWID with a stable HCV prevalence [38]. This could possibly result in a different pattern of HCV incidence with time since first injection compared with that found, for example, in an immature injecting epidemic with many recent initiates or where HCV prevalence among PWID is very high. It is possible that re-initiation to injecting after periods of cessation, for example during or after addiction treatment or imprisonment [39,40], may result in repeated short periods of elevated risk throughout a lifetime of injecting, similar to that which probably occurs at first initiation. Although this needs further investigation, the association with imprisonment, found here and previously noted [13,32], supports this possibility.

A related issue, although not significant, was that for the avidity marker, a higher proportion of the recent infections detected were among those who first injected more than 20 years ago (19% vs 7% for RNA). This might reflect misclassification bias and possibly be due to a small number of people with longer-term infections not developing HCV antibodies with high avidity. Reported false recency rates are less than 1–2% in individuals without HIV [14]. However, there may still be issues with reduced immune response in those with long-term HCV infection, similar to those seen in individuals living with HIV [20], but related to other co-morbidities.

Misclassification or non-capture of recent initiates would be expected to reduce the number of recent infections observed in this group, but as the vast majority of participants had been injecting for longer, this should have a relatively small impact on the overall incidence. Further, this is a minor point compared with the uncertainty of the window periods themselves [8,11,17,18] and does not preclude the use of recent infection markers to monitor trends in incidence. However, further work, for example using seroconversion panels, to improve understanding of the window periods would be helpful and is needed before routine clinical use of avidity testing.

Owing to the uncertainties in the window period for weak avidity, the combined model for incidence relied largely on the RNA data. Nevertheless, both markers have their merits: RNA is a reliable and well-understood marker of recent infection, but infrequently observed because of the short window period. The avidity-based marker may not be ideal for estimating absolute incidence, but has greater power to detect patterns according to risk factors or changes over time. By combining
both markers, we could identify risk factors for recent infection for which there was insufficient power – even in our relatively large sample – when using either marker alone. Future work could incorporate markers of recent infection and the FOI approach within a combined model. Although work is required to resolve a number of issues, such as the discrepancy between biological markers and FOI methods for injecting duration-specific risk, such modelling has the potential to simultaneously refine window period estimates and provide estimates of incidence with greater power.

The combined use of both markers of recent infection could reduce the need for very large sample sizes and costs. Power calculations using a simple simulation-based approach [41] indicate that a survey recruiting annually around 1,000 participants without an established infection, and where around 5% of these participants had one of the two markers of recent infection at baseline, there would be over 90% power of detecting a halving of incidence over 4 years, although a reduction by one third may not be detectable (56% power). If the RNA marker is used alone (ca.2% at baseline), only a reduction by two thirds would be reliably detected (power=86%) and detecting a halving of incidence is not guaranteed (power=54%). This is a general problem with detecting decreases in incidence as over time, the number of recent infections will become extremely low. Of course, a genuine decrease in incidence will also result in lower prevalence, especially in recent initiates to injecting; therefore a combined approach may be most useful for detecting the impact of TasP, NSP and OST, and in monitoring progress towards the global strategy target to reduce HCV incidence by 90% by 2030 [6]. However, the actual approach taken in a country or area will depend on the baseline hepatitis C incidence and the rate of decline expected. These approaches will usually provide a better insight into the extent of recent infections than data on new HCV diagnoses, as people may have been infected some time before their diagnosis and changes in diagnosis rates may not reflect underlying incidence but rather detection rates and testing practice, e.g. following temporal variations in the offer and uptake of diagnostic testing. However, monitoring incidence using the approaches applied here may not be practical among those populations and groups where the overall HCV incidence and risks are very low; very large sample sizes would be needed, and combination of methods may therefore be required.

Conclusion
Our findings indicate that the two biological approaches to estimating incidence identified associations with similar injecting risk behaviours and social and demographic profiles. This indicates that both have utility in monitoring incidence, although the short-lived nature of the states assessed by these two markers limits the use of only one marker, particularly when the incidence is not high. Meeting the hepatitis C elimination goals in the Global Health Sector Strategy for Hepatitis [6], will require an assessment of the impact on HCV transmission of interventions such as NSPs, OST and TasP that need to be delivered to PWID to achieve it, in the context of declining incidence. A robust measure of incidence among PWID could be provided by using the two biological markers examined here in combination.

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The NIHR HPRU in Blood Borne and Sexually Transmitted Infections Steering Committee: Caroline Sabin (Director), Anthony Nardone (PHE Lead), Catherine Mercer, Gwenda Hughes, Jackie Cassell, Greta Rait, Samreen Ijaz, Tim Rhodes, Kholoud Porter, Sema Mandal, and William Rosenberg. The views expressed in this publication are those of the authors and not necessarily those of the NHS, the National Institute for Health Research, the Department of Health or Public Health England.

Conflict of interest
None declared.

Authors’ contributions
All authors contributed to the writing of the paper. The writing was led by VH and analyses were undertaken by RH. The survey delivery was co-ordinated by VH, KC, FN, and MD, with laboratory work managed and co-ordinated by JVP and SI, and the incidence testing work undertaken by JS. VH, PV, LP, RH, JVP, SI and SM contributed with the design of the study and the analyses.

References


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Impact of pneumococcal conjugate vaccines introduction on antibiotic resistance of Streptococcus pneumoniae meningitis in children aged 5 years or younger, Israel, 2004 to 2016

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Background: Empiric treatment of pneumococcal meningitis includes ceftriaxone with vancomycin to overcome ceftriaxone resistant disease. The addition of vancomycin bears a risk of adverse events, including increased antibiotic resistance. We assessed antibiotic resistance rates in pneumococcal meningitis before and after pneumococcal conjugate vaccine (PCV) implementation. Methods: All pneumococcal meningitis episodes in children aged 5 years and younger, from 2004 to 2016, were extracted from the nationwide bacteremia and meningitis surveillance database. For comparison purposes, we defined pre-PCV period as 2004–2008 and PCV13 period as 2014–2016. Minimal inhibitory concentration (MIC) > 0.06 and > 0.5 μg/mL were defined as penicillin and ceftriaxone resistance, respectively. Results: Overall, 325 episodes were identified. Pneumococcal meningitis incidence rates declined non-significantly by 17%, comparing PCV13 and pre-PCV periods. Throughout the study, 90% of isolates were tested for antibiotic susceptibility, with 26.6%, 2.1% and 0% of isolates resistant to penicillin, ceftriaxone and vancomycin, respectively. Mean proportions (± SD) of meningitis caused by penicillin-resistant pneumococci were 40.5% ± 8.0% and 9.6% ± 7.4% in the pre-PCV and the PCV13 periods, respectively, resulting in an overall 83.9% reduction (odd ratio:0.161; 95% confidence interval: 0.059–0.441) in penicillin resistance rates. The proportions of meningitis caused by ceftriaxone resistant pneumococci were 5.0% ± 0.8% in the pre-PCV period, but no ceftriaxone resistant isolates were identified since 2010. Conclusions: PCV7/PCV13 sequential introduction resulted in > 80% reduction of penicillin-resistant pneumococcal meningitis and complete disappearance of ceftriaxone resistant disease. These trends should be considered by the treating physician when choosing an empiric treatment for pneumococcal meningitis.

Introduction

Bacterial meningitis is a major cause of morbidity and mortality in children worldwide, with Streptococcus pneumoniae being the leading cause of bacterial meningitis in up to 60% of cases [1,2]. Pneumococcal meningitis has high rates of long-term complications (e.g. behavioural/intellectual disorders, hearing loss and neurologic deficits), with mortality rates of up to 26% [2].

The early and rapid administration of antibiotics is crucial to increase survival and reduce morbidity in pneumococcal meningitis and the choice of empiric antibiotics should be based on the local epidemiology of antibiotic susceptibility, among other factors [3]. Antibiotic resistance definitions for pneumococcal meningitis are stricter than those for non-meningitis pneumococcal infections, with minimal inhibitory concentrations (MIC) cut-offs of 0.06 μg/mL for penicillin resistance and MIC > 0.5 μg/mL for ceftriaxone resistance, according to the Clinical and Laboratory Standards Institute (CLSI) [2,4].
Before the introduction of pneumococcal conjugate vaccines (PCVs) worldwide, an increase in pneumococcal antimicrobial (mainly penicillin) resistance had been observed [5-8], resulting in modifications of the empiric management of meningitis [8,9]. Initially, third generation cephalosporins (cefotaxime and ceftriaxone) became the standard therapy in children and subsequently, in the mid-1990s, reports of cephalosporin-resistant pneumococcal meningitis led to the recommendation of adding vancomycin as empiric therapy in suspected cases [3,10]. Therefore, the empiric treatment in cases of suspected pneumococcal meningitis currently includes intra-venous ceftriaxone and vancomycin pending culture results [10-12].

Due to its low penetration through the meninges, vancomycin is given at high doses (60mg/kg/day). The addition of empiric treatment with vancomycin may rarely result in side effects, such as hypersensitivity, nephrotoxicity (especially at high doses used to treat pneumococcal meningitis) and hearing loss [2,13]. Furthermore, using vancomycin increases the selection and exposure of bacteria to antibiotics and may accelerate the emergence of strains resistant to vancomycin, which is one of the antibiotics saved as last-resort treatment against bacteria resistant to other antimicrobial drugs [2,13-15].

In July 2009, the 7-valent pneumococcal conjugate vaccine (PCV7) was introduced to the Israeli national immunisation programme (NIP) and in November 2010, was replaced by the 13-valent pneumococcal conjugate vaccine (PCV13) [16]. By December 2012, the proportion of children who received two or more doses of PCV7 and PCV13 was greater than 95% [16]. Following PCV7/PCV13 sequential introduction, rates of pneumococcal meningitis and other invasive pneumococcal disease (IPD) caused by vaccine-serotype pneumococci substantially declined by ca 95% in children aged 5 years or younger [17]. In contrast, overall pneumococcal meningitis rates were reduced non-significantly by 27% and remaining cases mainly reflected an increased rate of non-vaccine serotype pneumococcal meningitis [17]. Besides the impact observed worldwide on IPD rates caused by vaccine-serotypes, including meningitis, PCV introduction in the United States (US) was also followed by a reduction in antibiotic resistance rate [18,19]. This reduction may have derived from the elimination of resistant vaccine strains and the increase in non-vaccine strains [17,18,20] that are more frequently antibiotic susceptible, as they were previously less exposed to antibiotic selection stress [18].

The empiric treatment of suspected pneumococcal meningitis with ceftriaxone and vancomycin both in Israel and in many settings worldwide [2,11] was rarely revisited following PCVs introduction. Nevertheless, vancomycin treatment might not be justified, if ceftriaxone resistance rates are shown to be low [3].

We assessed antibiotic resistance rates in pneumococcal meningitis episodes before and after PCV introduction to the Israeli NIP, in an attempt to optimise current treatment recommendations.

Methods
The ongoing, prospective, nationwide, population-based and active surveillance study, conducted by the Israeli Paediatric Bacteraemia and Meningitis Group, was initiated in 1989. The current report describes data spanning over a 12-year period (July 2004–June 2016). Incidence rates of pneumococcal meningitis are presented from July 2000, to allow better appreciation of secular trends and fluctuations.

The study was approved by the Institutional Ethics Committees of the participating medical centres.

Setting and study population
The study population comprised all children aged 5 years or younger in Israel. As of 2016, Israel had a population of ca875,000 children in this age group [21]. The study has been conducted in all 26 medical health centres routinely obtaining cerebrospinal fluid (CSF) cultures from children [16,22], no CSF cultures are obtained outside these centres, enabling us to collect the majority of culture-confirmed pneumococcal meningitis cases in Israel.

### Table 1
Number of pneumococcal meningitis episodes and incidence rates in children aged 5 years or younger, Israel, July 2000–June 2016

<table>
<thead>
<tr>
<th>Time period</th>
<th>Pneumococcal meningitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of episodes</td>
</tr>
<tr>
<td>July 00–June 01</td>
<td>26</td>
</tr>
<tr>
<td>July 01–June 02</td>
<td>30</td>
</tr>
<tr>
<td>July 02–June 03</td>
<td>48</td>
</tr>
<tr>
<td>July 03–June 04</td>
<td>26</td>
</tr>
<tr>
<td>July 04–June 05*</td>
<td>25</td>
</tr>
<tr>
<td>July 05–June 06*</td>
<td>27</td>
</tr>
<tr>
<td>July 06–June 07</td>
<td>33</td>
</tr>
<tr>
<td>July 07–June 08</td>
<td>27</td>
</tr>
<tr>
<td>July 08–June 09</td>
<td>38</td>
</tr>
<tr>
<td>July 09–June 10</td>
<td>29</td>
</tr>
<tr>
<td>July 10–June 11</td>
<td>23</td>
</tr>
<tr>
<td>July 11–June 12</td>
<td>27</td>
</tr>
<tr>
<td>July 12–June 13</td>
<td>33</td>
</tr>
<tr>
<td>July 13–June 14</td>
<td>27</td>
</tr>
<tr>
<td>July 14–June 15</td>
<td>28</td>
</tr>
<tr>
<td>July 15–June 16*</td>
<td>28</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IR (95% CI)</th>
<th>PCV 13 vs pre-PCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>0.83 (0.60–1.15)</td>
</tr>
</tbody>
</table>

CI: confidence interval; IRR: incidence rate ratio; NA: not available; PCV: pneumococcal conjugate vaccine.

* Period termed as ‘pre-PCV period’ defined as 2004–2008.

* Period termed as ‘PCV 13 period’ defined as 2014–2016.
Local investigators in each centre responded to a monthly distributed questionnaire sent by the principal investigator at the study headquarters.

Case definitions
A pneumococcal meningitis episode was defined as an illness during which *S. pneumoniae* was isolated from either CSF or from blood with laboratory signs suggestive of meningitis (e.g., CSF pleocytosis). Non-culture diagnoses (polymerase chain reaction, antigen testing, Gram stain results or clinical diagnosis) were excluded. In Israel, < 5% of all meningitis episodes are diagnosed by non-culture methods.

Antibiotic susceptibility testing
According to the National Committee for Clinical and Laboratory definitions for antibiotic susceptibility in pneumococcal meningitis, isolates with a MIC of $> 0.06 \mu g/mL$ for penicillin were determined as penicillin resistant. Isolates with an MIC $> 0.5 \mu g/mL$ for ceftriaxone were defined as ceftriaxone resistant [2,13].

### Uptake of pneumococcal conjugate vaccines

PCV7 NIP was initiated in July 2009 with a catch-up campaign in children aged 2 years or younger [17,23]. In November 2010, PCV13 replaced PCV7 without further catch-up.

Vaccine uptake evaluation methods were previously described [16,17]. By June 2011 and December 2012, ca 80% and ca 90%, respectively, of 7–11 month old children received two or more doses of PCV7 and/or PCV13 and ca 95% received two or more PCV13 doses by June 2014 and June 2015.

By June 2011 and December 2012, 36% and 87%, respectively, of children aged 24–35 months, received three or more PCV7/PCV13 doses and $> 90\%$ received three or more doses of PCV13 by June 2014 and June 2015.

### Data analysis

Annual (July to June) incidence rates were calculated as the number of CSF positive culture cases divided by the population.
total population at risk during each year of the study [21].

For episodes in which the serotype and/or serogroup were missing, a detailed extrapolation was conducted, as described elsewhere [22]. Briefly, the proportion of episodes attributed to each serotype-group (vaccine type and non-vaccine type) was assigned from the age- and ethnicity-specific strata, assuming serotype data were missing at random.

Since 2009–2010, the proportion of isolates with serotype determination increased to >95% from ca 60% in the pre-PCV period (2004–2008) [22].

To assess changes in meningitis incidence and proportions of antibiotic resistance (penicillin MIC > 0.06; ceftriaxone MIC > 0.5) of all isolates, we used annual rates. Data are presented for all study years (2004–2016). In addition, we compared rates in the pre-PCV period (July 2004–June 2008) and the last two study years (July 2014–June 2016) as the PCV13 period.

Incidences were calculated using the birth cohorts born in Israel, according to the Israeli Central Bureau of Statistics reports [21]. Incidence rate ratios (IRRs) and 95% confidence intervals (CIs) were calculated for meningitis rates.

Proportions of antibiotic resistance of all isolates and odds ratios (ORs) with 95% CI were calculated. P values of dynamics in proportions of antibiotic resistance were calculated.

Data were analysed with SPSS 23.00 software. Univariate analyses were conducted using two-tailed chi-squared test or Student t-test, where appropriate. A p value < 0.05 was considered statistically significant.

## Results

During the study period, 325 pneumococcal meningitis episodes were identified in children aged 5 years or younger. The mean age was 14.5 ± 13.7 months, and median age was 10.1 months, with 54.8% (n = 178) of children aged 12 months of less. Of the 325 episodes, 53.2% (n = 173) were in males.

Of all 325 isolates, 289 (88.9%) and 291 (89.5%) were tested for penicillin and ceftriaxone susceptibility, respectively. Overall, throughout the study, 77 (26.6%) isolates were penicillin-resistant and six (2.1%) isolates were ceftriaxone-resistant. All (n = 290) tested isolates were susceptible to vancomycin.

### Pneumococcal meningitis rates dynamics

Following PCV13 introduction, meningitis rates declined non-significantly by 17% (IRR: 0.83; 0.60–1.15) comparing the PCV13 and the pre-PCV periods (Table 1, Figure 1).

### Proportions of penicillin-resistant isolates

In the pre-PCV period, proportions of meningitis caused by penicillin-resistant pneumococci were 40.5% ± 8.0% (Table 2, Figure 2). In the PCV13 period, these proportions significantly declined by 83.9% (OR = 0.16; 0.06–0.44) and were 9.6% ± 7.4%. Similarly, a significant declining trend of proportions of penicillin-resistant

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### Table 2

Proportions of penicillin-resistant and ceftriaxone-resistant pneumococcal meningitis of all isolates in children aged 5 years and younger, Israel, July 2004–June 2016

<table>
<thead>
<tr>
<th>Time period</th>
<th>Penicillin MIC &gt; 0.06 μg/mL</th>
<th>Ceftriaxone MIC &gt; 0.5 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of isolates</td>
<td>Percent</td>
</tr>
<tr>
<td>July 04–June 05 a</td>
<td>7/22</td>
<td>31.8</td>
</tr>
<tr>
<td>July 05–June 06 a</td>
<td>11/22</td>
<td>50.0</td>
</tr>
<tr>
<td>July 06–June 07 a</td>
<td>14/32</td>
<td>43.8</td>
</tr>
<tr>
<td>July 07–June 08 a</td>
<td>8/22</td>
<td>36.4</td>
</tr>
<tr>
<td>July 08–June 09</td>
<td>9/34</td>
<td>26.5</td>
</tr>
<tr>
<td>July 09–June 10</td>
<td>10/23</td>
<td>43.5</td>
</tr>
<tr>
<td>July 10–June 11</td>
<td>2/22</td>
<td>9.1</td>
</tr>
<tr>
<td>July 11–June 12</td>
<td>5/25</td>
<td>20.0</td>
</tr>
<tr>
<td>July 12–June 13</td>
<td>2/11</td>
<td>18.2</td>
</tr>
<tr>
<td>July 13–June 14</td>
<td>4/26</td>
<td>15.4</td>
</tr>
<tr>
<td>July 14–June 15 b</td>
<td>1/23</td>
<td>4.3</td>
</tr>
<tr>
<td>July 15–June 16 b</td>
<td>4/27</td>
<td>14.8</td>
</tr>
</tbody>
</table>

OR (95% CI)

PCV 13 vs pre PCV

|                      | NA            | 0.16 (0.06–0.44) | NA | NA |

CI: confidence interval; MIC: Minimal inhibitory concentration; NA: not available; OR: odds ratio; PCV: pneumococcal conjugate vaccine.

a Period termed as ‘Pre-PCV period’ defined as 2004–2008.

b Period termed as ‘PCV 13 period’ defined as 2014–2016.
Proportions of ceftriaxone-resistant pneumococcal meningitis within pneumococcal isolates is presented in supplement.

**Proportions of ceftriaxone-resistant pneumococcal isolates in pneumococcal meningitis**

In the pre-PCV period, proportions of meningitis caused by ceftriaxone-resistant pneumococci were 5.0% ± 0.8% (Table 2, Figure 3). Following PCV13 introduction, these proportions declined to 0.0%. No cases of ceftriaxone resistant isolates were observed since July 2010.

**Proportions of PCV13 serotypes (VT13), and non-VT13 pneumococcal isolates in antibiotic-resistant meningitis**

Of 40 penicillin-resistant isolates in the pre-PCV period, 26 had a known serotype. Since July 2009, all 28 penicillin resistant isolates had known serotypes. Of 26 resistant isolates in the pre-PCV period, 21 were VT13, while in the PCV13 period, none (0/5) of the resistant isolates were VT13 (Figure 4).

Within the non-VT13 group, penicillin resistance rates declined from 33.3% in the pre-PCV period to 10.4% in the PCV13 period (p = 0.049).

For the six ceftriaxone resistant isolates (all before July 2010), four isolates had a known serotype; all three isolates from the pre-PCV period were VT13 and the one isolate from the year 2009–2010 was non-VT13.

**Discussion**

The sequential introduction of PCV7/PCV13 into the Israel NIP resulted in an 80% decline in the incidence of antibiotic-resistant pneumococcal meningitis, even though the overall pneumococcal meningitis incidence rates did not decline significantly; reflecting the near elimination of disease caused by vaccine serotypes.

Before PCV implementation, vaccine-serotypes were the most successful serotypes in nasopharyngeal colonisation and therefore the most frequent pathogens causing pneumococcal diseases [24]. This frequent and relatively prolonged colonisation and involvement...
in disease resulted in continuous antibiotic pressure (selection for an antibiotic-resistant strain deriving from frequent or prolonged exposure to antibiotics) due to the high antibiotic consumption in young children and thus leading to the emergence of antibiotic resistance and multi-drug resistance [25].

In the pre-PCV era, an increased rate of antibiotic non-susceptibility in pneumococcal meningitis was observed in many sites worldwide [5-8,13,14,26,27]. This increase supports the well-known phenomenon of increased antibiotic resistance following the continuous usage of antibiotic, driven by selective pressure [24]. In southern Israel, seasonal variations in antibiotic resistance among otitis media pneumococcal isolates were associated with substantial variations in antibiotic consumption [28]. Furthermore, increased resistance to multiple antibiotics among nasopharyngeal carried pneumococcal isolates was associated with increased azithromycin consumption [29]. A recent study from southern Israel showed reduction in overall antibiotics dispatched prescription rates in children following PCV7/PCV13 sequential introduction [30], further supporting our hypothesis. Following PCV7 introduction, a substantial decrease in pneumococcal meningitis caused by PCV7 serotypes was observed, along with an increase in disease caused by non-vaccine serotypes, including strains non-susceptible to antibiotics [12,18,19,31]. Similarly, following PCV13 introduction, a substantial decline in PCV13-serotypes disease rates, along with an increase in non-VT13 serotypes were observed [17,23,32-34].

The reduction in disease caused by VT13 serotypes is accompanied by a reduction in the carriage of vaccine serotypes [24] resulting in decreased 'antibiotic pressure'. In the pre-PCV era, vaccine-serotypes were the main pneumococcal serotypes carried and responsible for causing disease; consequently, these were the main serotypes exposed to antibiotic pressure. It is not surprising, therefore, that these serotypes were the main strains exhibiting antibiotic resistance in the pre-PCV era. In contrast, non-vaccine serotypes were less exposed to antibiotic pressure and with the near elimination of vaccine-serotypes, the main burden of antibiotic resistance was eliminated. Nevertheless, it

### Figure 3

Proportions of ceftriaxone-resistant pneumococcal meningitis of all isolates in children aged 5 years and younger, Israel, July 2004–June 2016 (n = 291)

PCV: pneumococcal conjugate vaccine.

Ceftriaxone-resistant pneumococcal meningitis = minimal inhibitory concentration > 0.5 μg/mL.

‘Pre-PCV period’ defined as 2004–2008 and ‘PCV 13’ period defined as 2014–2016.
is important to recognise the possibility of new emerging antibiotic resistant strains among non-vaccine serotypes, deriving from the time elapsed since PCV13 introduction and increased exposure of the now predominating non-vaccine serotypes to antibiotic pressure. Indeed, following PCV7 introduction in the US, rapid replacement with penicillin-non-susceptible non-vaccine serotypes was observed [35]. However, when the overall impact on antibiotic resistance was evaluated, the outcome was positive.

Currently in Israel, treatment of pneumococcal meningitis in children involves empiric addition of vancomycin to ceftriaxone. It was previously suggested that in the epidemiologic setup of low ceftriaxone resistance (< 1%), it may be suitable to treat suspected pneumococcal meningitis cases empirically with ceftriaxone only, without adding vancomycin to the treatment regimen [3]. With this in mind, it may be suitable to recommend ceftriaxone only as an empiric treatment for pneumococcal meningitis in Israel (and possibly other settings where ceftriaxone resistance is low) assuming there is suitable ongoing surveillance. A possible advantage to removing vancomycin from the empiric treatment of meningitis would be in the reduction of potential side effects to the drug, including the emergence of strains resistant to vancomycin.

Continuous surveillance is needed to better understand and track antibiotic resistance rates, as well as identifying emerging resistant serotypes. In addition, surveillance could help to possibly developing new, broader (higher valency or protein based), pneumococcal vaccines.

The major strengths of our study include the utilisation of prospective, active and population-based methodology, as well as a large number of cases. The relatively long time period elapsed since PCV13 introduction to the Israeli NIP (6 years) also enables a more accurate evaluation of PCV13 impact. The main limitation of our study is the relatively high rate of undetermined serotypes early in the pre-PCV period. In recent years, however, the rate of extrapolated serotypes has dropped to < 5% of all isolated pneumococci. Notably, while in the pre-PCV period only 65% of penicillin-resistant isolates had a known serotype, this proportion decreased to 0% and has remained so since July 2009. However, while incomplete data on serotypes is a limitation, testing of penicillin resistance was done for ca. 90% of
isolates providing a comprehensive picture of penicillin resistance dynamics.

An additional limitation lies in the analysis of IRRs and ORs comparing grouped study years according to PCVs uptake (i.e. pre-PCV, PCV7 and PCV13 period) and the relatively small sample size in each year. Nevertheless, incidence rates for overall pneumococcal meningitis and antibiotic resistance proportions among all isolates and serotype sub-groups (VT13, non-VT13) are presented for each individual year. Additionally, chi-square for linear trend was calculated for penicillin resistance rates (proportions) and showed statistically significant reduction.

In summary, PCV7/PCV13 sequential introduction resulted in >80% reduction of penicillin-resistant incidence rates of pneumococcal meningitis, in parallel with the disappearance of ceftriaxone resistant disease in Israeli children aged 5 years or younger. These trends should be considered by physicians when choosing an empiric treatment for pneumococcal meningitis.

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Conflict of interest

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References


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